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13. ABSTRACT (Maximum 200 Words) The objective of the present research is to determine whether there is a coherent body of evidence implicating oxidative damage in the pathogenesis of Parkinson's Disease and the MPTP model of Parkinsonism. We carried out initial studies in the Parkinsonian Syndrome known as Progressive Supranuclear Palsy. These studies showed that there is significant increase in lipid peroxidation in the subthalamic nucleus. We developed a novel column switching assay for measurement of an oxidative marker of damage to DNA in human body fluids. We found that both overexpression of manganese superoxide dismutase, a major free radical scavenging enzyme, as well as Bcl2 and a dominant negative inhibitor of interleukin converting enzyme all significantly attenuate MPTP induced dopaminergic neurotoxicity. This is accompanied by reductions in markers of oxidative stress. We also found that a number of therapeutic interventions, which may modulate oxidative stress, are effective in the MPTP model. We found that several novel free radical spin traps attenuate MPTP induced neurotoxicity and also attenuate oxidative damage. Lastly, we found that oral administration of creatine or cyclocreatine are neuroprotective against MPTP neurotoxicity. The studies to date, have made significant progress on the original aims of the proposal.			
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FOREWORD

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N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

M I Hunt Beal 10/20/99

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5. INTRODUCTION

Oxidative stress from indogeneous and enzogenous oxidants has been implicated as a major cause of Parkinson's Disease. The subject of the present research is to:

1. Investigate whether there is coherent evidence of increased oxidative damage to proteins, lipids and DNA in postmortem tissue of patients with Parkinson's Disease.
2. To develop novel HPLC based assays for quantitation of products of oxidative damage in human CSF, plasma and urine samples and to apply these biomarkers to study whether they are altered in patients with Parkinson's Disease.
3. To determine whether oxidative stress plays a key role in neuronal death, which occurs in the MPTP model of Parkinson's Disease.

In particular, we were going to examine whether transgenic mice with alterations in free radical scavenging enzymes or which overexpress Bcl2 are resistant to MPTP neurotoxicity. We were also going to examine whether free radical spintraps, neuronal nitric oxide synthase inhibitors and creatine can block MPTP neurotoxicity.

6. BODY

The program unfortunately suffered the loss of one of its coinvestigators, Dr. John B. Penney Jr., who died unexpectedly on January 31, 1999. His position in the project has been filled by Dr. Anne B. Young, Chief of Neurology at Massachusetts General Hospital, and the studies are proceeding.

We have now supplied a letter from Dr. Young indicating her willingness to participate, a CV, and a statement of her other support. With regard to the concerns of the absence of recognition of support by USAMRMC in some of the publications in the appendix this was partly due to a delay in funding. We have now cited USAMRC support in all new publications as well as several reviews, which cite the data.

Objective #1 – to determine whether there is coherent evidence of increased oxidative damage to the protein, lipid and/or DNA fractions in postmortem human brain tissue of patients with PD as compared to age-matched controls.

With regard to the contractual issues raised in the report of our contracting officer representative, we are continuing to collect postmortem brain tissue. We have carried out preliminary studies of 3-nitrotyrosine and protein carbonyls in postmortem tissue but there was a large variance in the measurements and we are attempting to collect further samples. We now suspect that nitro-gammaglutathione may be a more reliable assay for nitration and we are working on validating a reliable assay.

We have carried out initial studies of postmortem brain tissue of patients with Progressive Supranuclear Palsy. These patients develop a parkinsonian syndrome. We examined a well-established marker of oxidative damage to lipids in the subthalamic nucleus and cerebellum from 11 patients with Progressive Supranuclear Palsy and 11 age-matched controls using sensitive HPLC techniques (Albers et al, 1999). In Progressive Supranuclear Palsy, we found a significant increase in tissue malondialdehyde levels in the subthalamic nucleus when compared to the age-matched control group. By contrast, there were no significant differences between tissue malondialdehyde content in cerebellar tissue from the same Progressive Supranuclear Palsy in age-matched control cases. We concluded from this that lipid peroxidation may play a role in the pathogenesis of Progressive Supranuclear Palsy.

We have commenced studies using immunocytochemical assays for oxidative damage in Parkinson's Disease postmortem tissue. In the past year, we have assembled a panel of human brain tissue from normal midbrain as well as a smaller number of samples from patients with Parkinson's disease. We have conducted preliminary trials of staining for MDA and HNE in rodent tissue, to optimize staining parameters. Once all the tissues are in hand, the immunohistochemical studies should proceed rapidly. We also intend to utilize

new techniques including in situ hybridization to look for oxidative damage in Parkinson's Disease postmortem tissue.

As described in the proposal, we have constructed in situ hybridization probes for MnSOD, and for comparison, Cu/ZnSOD. These have been successfully hybridized to human brain, and produce a robust signal. We are working to expand the number of cases labeled, and then will undertake the quantitative analysis as proposed. Probes to the other markers (glutathione peroxidase, and bcl-2) are under development and should be available in the next few months for similar studies.

In addition, we have successfully employed Laser Capture Microdissection to isolate single neurons from human substantia nigra. We have confirmed by PCR the recovery of UCLH1 mRNA, a key dopaminergic marker. We have extended this method to larger numbers of neurons (>10,000) in a single experiment, and are preparing to hybridize the isolated mRNA to DNA microarrays in the next few weeks. Following these preliminary studies, we plan to employ this powerful method to the hypothesis that differential gene expression confers selective vulnerability to substantia nigra neurons.

Objective #2: To develop novel HPLC based assays for quantitation of products of oxidative damage in human CSF, plasma and urine samples and to apply these biomarkers to study whether they are altered in patients with Parkinson's Disease.

We have made marked progress in developing novel assay to measure 8-hydroxy 2-deoxyguanosine in urine, plasma and CSF as well as other biological matrices. These studies have been recently published by (Bogdanoff et al;1999). We developed a liquid chromatography electrochemical column switching system based on the use of a unique pairing selectivity of porous carbon columns that allow routine active measurement of 8-hydroxy-2-deoxyguanosine in numerous biological matrices. We obtained baseline concentrations in both human and animal tissue as well as body fluids. We utilized this system in approximately 3600 samples using internal quality control and external blind testing to determine long-term accuracy. The methods are very reliable and accurate. This is a unique advance in the measurement of oxidative damage for human body fluids. We intend to utilize this assay to further examine Parkinsonian patients as we had initially proposed.

The reviewer notes that the OH⁸dG assay to be used for objective 1 has been validated, but that we had originally stated it was well established. It was well established however, in examining a large number of body fluid samples, and in particular urine samples, we became aware of interferences. We, therefore, established and validated a new highly reliable methodology using column switching. This gives us unequivocally reliable and precise measurements in human body fluids. With regard to further progress in objective 2, we have established an assay for nitro-gammatocopherol for use in human body fluids. Further validation in studies are underway. We are in the process of collecting well-validated plasma and urine samples from patients and controls for the measurements.

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Objective #3: to determine whether oxidative stress plays a role in the neuronal death in the MPTP model of Parkinson's disease, and whether the same stress may be operating in human nigral neurons.

We have carried out a number of studies which have examined MPTP neurotoxicity in transgenic mice as well as the effects of a number of therapeutic interventions. We used mice that overexpressed manganese superoxide dismutase (Klivenyi et al, 1998). There is substantial evidence implicating free radical production from mitochondria in the neurotoxicity of MPTP. Manganese superoxide dismutase is the primary antioxidant enzyme found within mitochondria. We studied mice, which have approximately a 50% increase in total manganese SOD activity in brain homogenates. We also localized manganese SOD to the mitochondria and neurons. We found that MPTP toxicity was significantly attenuated in the mice, which overexpressed manganese superoxide dismutase. These mice showed a three-fold greater dopamine concentration than controls following MPTP administration. There are no alterations in MPP+ levels suggesting that the effects were not due to altered metabolism of MPTP. A significant increase in 3-nitrotyrosine levels was seen in littermate controls but was not observed in transgenic mice overexpressing manganese SOD. These studies, therefore, provide further evidence implicating mitochondrial dysfunction and oxidative damage in the pathogenesis of MPTP toxicity.

We also carried out studies of transgenic mice, which overexpressed Bcl2. Bcl2, (Yang et al, 1998). Bcl2 is one of the primary proteins, which inhibits apoptotic cell death. It also has been demonstrated to exert antioxidant effects in vitro and we recently demonstrated that this was also the case in vivo. We investigated MPTP neurotoxicity in both Bcl2 overexpressing mice as well as littermate controls. We initially determined that there were no alterations in

dopamine cell counts in the substantia nigra of the Bcl2 overexpressing mice. MPTP induced depletion of dopamine and loss of 3H-Mazindol binding were significantly attenuated in Bcl2 overexpressing mice. Protection was more profound with an acute dosing regimen than with a daily administration of MPTP over five days. This was a surprising observation in that it was thought that more chronic administration is more likely to result in apoptotic cell death. MPP+ levels after MPTP administration was similar in Bcl2 overexpressing mice and littermate controls. Bcl2 also blocked the MPP+ induced activation of caspases and MPTP induced increases in free 3-nitrotyrosine levels were blocked in Bcl2 overexpressing mice. These studies, therefore, further implicate both oxidative damage as well as apoptosis in the neurotoxicity of MPTP.

Similarly we also looked at whether transgenic mice expressing a dominant negative mutant interleukin 1 β converting enzyme show resistance to MPTP toxicity. MPTP results in a significant depletion of dopamine, DOPAC and HVA in littermate control mice which was completely inhibited in the mutant interleukin-1 β converting enzyme mice (Klivenyi et al, 1999) there was also significant protection against MPTP induced depletion of tyrosine hydroxylase immunoreactive neurons. There is no alteration MPTP uptake or metabolism. These results, therefore, provide further evidence that apoptotic cell death as well interleukin converting enzyme may play an important role in the neurotoxicity of MPTP.

We also examined whether there was evidence of increased oxidative damage in the substantia nigra of baboons following administration of MPTP (Ferrante et al, 1999). We found that there was indeed a significant increase as detected by immunocytochemistry. This increase in 3-nitrotyrosine within the substantia nigra dopaminergic neurons was markedly attenuated by administration of the neuronal nitric oxide synthase inhibitor 7 nitroindazole.

We examined whether a number of novel free radical spintraps can inhibit MPTP neurotoxicity. We examined whether treatment with the cyclic nitron free radical spintrap MDL 101, 002 will protect against MPTP induced depletion of dopamine and its metabolites (Matthews et al, 1999b). MDL 101, 002, significantly attenuated MPTP induced dopaminergic neurotoxicity. It also significantly attenuated MPTP induced increases in striatal 3-nitrotyrosine concentrations. The free radical spin trap tempol also produced significant protection against MPTP neurotoxicity. These findings provide further evidence that free radical spintraps produced neuroprotective effects in vivo suggests that they might be useful in treating parkinsonism in man.

Lastly, we examined whether creatine and cyclocreatine administration can attenuate MPTP neurotoxicity (Matthews et al, 1999a). Oral supplementation with creatine or cyclocreatine, which are substrates for creatine kinase, may increase phosphocreatine or cyclophosphocreatine and buffer against ATP depletion and thereby exert neuroprotective effects. We found that oral supplementation with either creatine or cyclocreatine produced significant protection against MPTP induced dopamine depletions in mice. Creatine also protected against MPTP induced loss of nissl in tyrosine hydroxylase

immunostaining neurons in the substantia nigra. Creatine and cyclocreatine had no effects on the metabolism of MPTP to MPP+ in vivo. These studies suggest a further novel therapeutic approach for MPTP and by implication parkinsonism.

Over the past year, we have completed 2 further studies which are now accepted for publication and in which we have cited Department of Defense support for the studies. We found that creatine administration protects against malonate and NMDA toxicity and creatine with nicotinamide has additive effects against malonate-induced lesions.

We have also studied the neuroprotective effects of a highly specific inhibitor of nNOS, ARR 17338. This inhibitor has the advantage that it has no potentially confounding effects on MAOB, such as had been suggested for 7-nitroindazole. This nNOS inhibitor exhibited dose-dependent protection against MPTP induced dopaminergic neurotoxicity.

Although we have completed many of the studies in the original schedule of work for specific aim 3. The results have led to new further related experiments, which we intend to pursue over the coming year. For instance, we have obtained a new strain of manganese superoxide dismutase overexpressing mice from Charles Epstein in which we will examine MPTP toxicity. Similarly, we have obtained mice with a 50% deficiency of manganese superoxide dismutase.

Plans for Years 2-4 of the Project.

The plans for the project have been modified because of several unanticipated events. Both the unexpected death of Dr. John B. Penny, Jr., one of the project leaders, as well as the relocation of project to Cornell University have unquestionably delayed some aspects of the study, and lead to reconsideration of the remaining goals. The most important event, however, has been the remarkably rapid evolution of new technology for studying injury and protective mechanisms in human brain disease.

Although the project is now located at Cornell University, the investigators at Massachusetts General Hospital are willing to continue to participate through a subcontract arrangement. The team at Massachusetts General would be directed by Dr. Anne B. Young, M.D., Ph.D. Additional personnel would include Dr. David G. Standaert, M.D., Ph.D, Dr. Sarah J. Augood, Ph.D and Dr Ippolita Cantuti-Castelvestri Ph.D. (see attached CV's, and letter from Dr. Young). The goals of the Massachusetts General Hospital investigators would be:

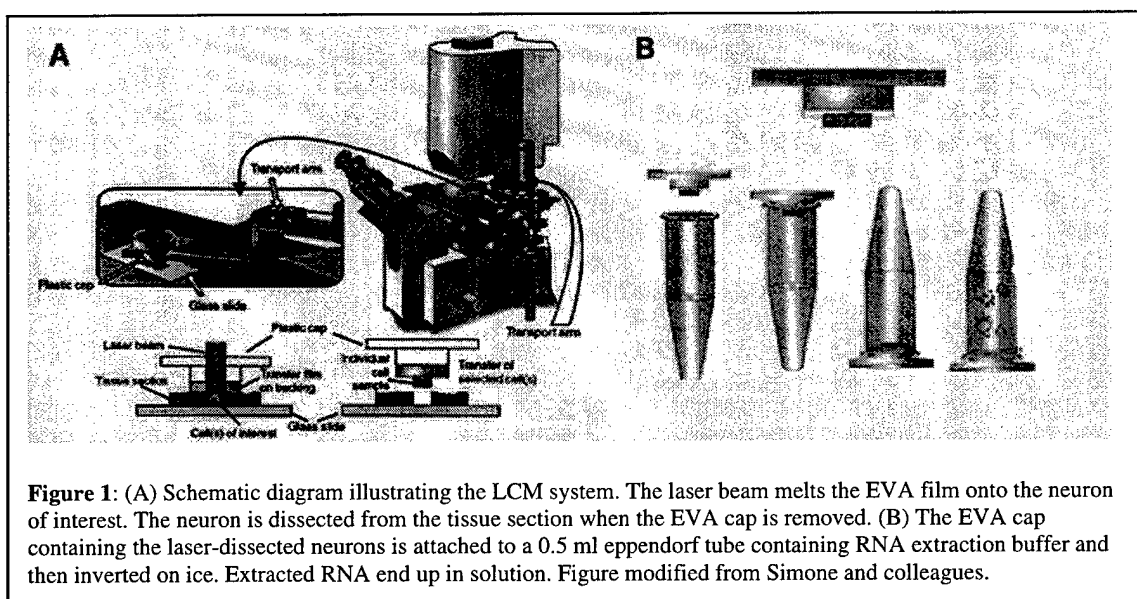
- 1) to support the efforts of the Principal Investigator by conducting the histological studies on human postmortem tissue required under Objective 1.
- 2) To pursue the goal of Objective 3, identifying the factors underlying the selective vulnerability of dopamine neurons, both through the use of dual-label in situ hybridization as originally described, and through the application of two new and powerful technologies, *laser capture microdissection* and *genetic array profiling*.

Laser capture microdissection (LCM):

Laser-capture microdissection (LCM) is a remarkable new technology which has become available for use within the last year. With this technology it is now possible to examine the molecular basis of cellular function, for example cellular resistance or vulnerability to excitotoxicity or disease, by micro-dissecting discrete cell populations. The populations for study can be selected on anatomic criteria, for example the dorsal or ventral tier of the substantia nigra, or can be identified by immunohistochemical methods. This later approach would permit selection of neuronal populations on neurochemical criteria, or by the presence of oxidative markers. After extract of RNA from the target cells, the molecular signature of the cell populations can be identified and compared using either conventional PCR based methods, or gene array profiling.

The technique of LCM was initially developed within the cancer field to facilitate the segregation of benign and malignant cells and has only recently been applied to the study of the CNS. Essentially, a 3-8 μm thick frozen section is rapidly processed for routine histology using RNase-free reagents. The section is then placed on an inverted microscope and overlaid with a ethylene vinyl acetate (EVA) transfer cap containing infrared absorbing dye. Neurons for laser-dissection are then selected within the laser beam (adjustable diameter 7-30 μm) and a 50 ms laser pulse melts the EVA onto the targeted neuron where it solidifies. This LCM process can then be repeated hundreds of times with each cap. Selected EVA-impregnated neurons are finally harvested when the cap is removed from the tissue section: only the selected neurons are present on the EVA cap. The cap is then fitted onto a sterile 0.5 ml eppendorf tube containing RNA

extraction buffer (guanidine isothiocyanate/ β -mercaptoethanol), inverted and placed on ice. Finally, the eppendorf tube and EVA cap are microcentrifuged and the cap removed. The cellular contents of the selected neurons are then in solution. A schematic illustrating this exciting new technology is shown in **Figure 1**. The potential of this exciting new technique is enormous and has recently been used to study of the development and physiology of segregated populations of immune cells (Alizadeh et al., 2000), renal cells (Kohda et al., 2000) and benign and malignant prostatic epithelia cells (Ornstein et al., 2000).



Genetic Array Profiling

At the time the original proposal for our study was prepared, the principal method of studying gene expression in particular populations of neurons in the brain was in situ hybridization. This remains the "gold standard" for quantification of cellular expression levels. However, a limitation of this method is that only a single mRNA can be studied at a time. Thus, it is not well suited to exploratory studies that seek to identify genes not previously known to be involved in a particular form of neural injury or disease.

Within the last year, microarray techniques have become available which allow the measurement of mRNA levels for hundreds or thousands of genes in a single experiment. Several different techniques are currently available including commercial arrays prepared on membranes, containing several hundreds or thousands of genes deposited in a defined array (manufactured by Research Genetics, Clontech Inc.) or silicon-based arrays manufactured by Affymetrix Inc. The investigators at Massachusetts General Hospital have experience with each of these new technologies, as described below.

Use of New Technologies in Furtherance of the Scientific Aims of the Project.

A fundamental goal of the project is to identify the role of oxidative stress in the selective vulnerability of dopamine neurons in Parkinson's disease. As noted in the original proposal:

“A characteristic feature of PD is that there is a marked depletion of the neurotransmitter dopamine. The severity of injury to dopamine neurons varies greatly in different areas of the brain. The disease process begins in the cell islands of the ventral tier of the substantia nigra pars compacta (SNc) and then moves to the ventral tier matrix, third to the dorsal tier of SNc, fourth to pars lateralis and then to paranigral nucleus and the rest of the midbrain dopamine neurons” (pg 11, para 4).

We proposed to address the basis for this selective vulnerability through measurements of:

“the levels of gene expression for neuroprotectant molecules such as glutathione peroxidase, manganese dependent superoxide dismutase, and bcl-2 at the individual neuron level in postmortem human brain” (pg 12, para 1)

At the time the proposal was prepared, the dual-label in situ hybridization technique was the only method available to perform these measurements. Laser capture microdissection and array technology are vastly more powerful techniques which allow this goal to be addressed in a far more comprehensive manner. Rather than simply studying the expression of three genes which may be involved in the selective vulnerability of dopamine neurons, we can dissect neurons from each of the regions of the SNc and analyze in a single experiment the expression of thousands of genes. This approach serves not merely to confirm the existing models of the mechanisms of oxidative injury, but to generate substantial new knowledge about the mechanisms of selective vulnerability to oxidative stress.

Preliminary data supporting the technical capabilities

We have access to a PixCell II Laser Capture Microdissection system from Arcturus located at the Massachusetts General Hospital. We have used this system to laser dissect pigmented DA neurons from fresh frozen cryostat sections of

the human SNpc. Neurons were collected onto CapSure™ polymer caps (**Figure 2**).

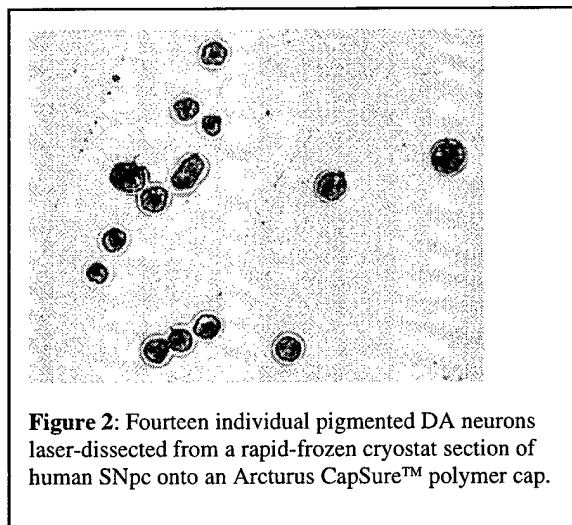
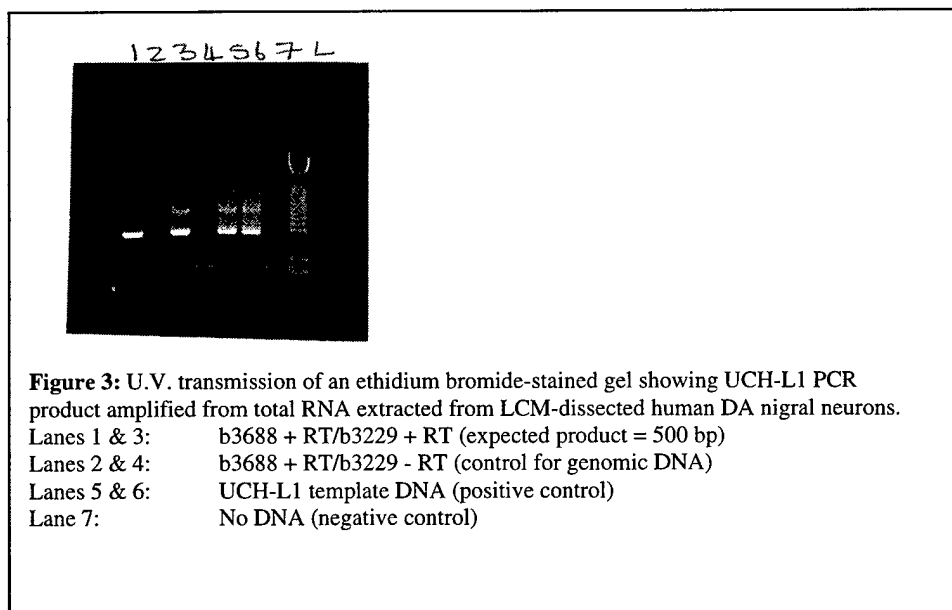


Figure 2: Fourteen individual pigmented DA neurons laser-dissected from a rapid-frozen cryostat section of human SNpc onto an Arcturus CapSure™ polymer cap.



Gene expression profiling using microarray cDNA membranes

Using methods for RNA extraction and RT-PCR established previously we have extracted intact mRNA from about 1,000 laser-dissected human DA neurons from 2 human brains (b3688 and b3229) provided by the Harvard Brain Tissue Resource Center. For each brain, the mRNA pool was divided in two and one sample incubated with Superscript II reverse transcriptase (to generate cDNA). The untreated mRNA pool serves as a negative control for contaminating genomic DNA. An aliquot of each sample was then used as template to PCR for UCH-L1 transcripts (**Figure 3**), a mRNA enriched in human nigral DA neurons.

As expected a major PCR product of the predicted size (500 bp) is detected only in samples treated with reverse transcriptase (Lanes 1 and 3).

Human "Named Genes" DNA microarray filters (Research Genetics GF-211) contain 4,200 known cDNA fragments which have been sequenced by Research Genetics and verified to be correct. Most cDNAs contain approximately 300 bp of 3'-coding sequence. We have already screened these filters with a ^{33}P -labelled DNA pool generated from LCM-dissected dopamine neurons from a post-mortem human brain (b3688). The hybridized filter was opposed to a phosphor-imager screen for 48 hrs and the resulting image analyzed using PathwaysTM, Research Genetics software. As can be seen in **Figure 4** a restricted profile of mRNA expression is observed, illustrating the "molecular" signature for this LCM-derived human brain sample.

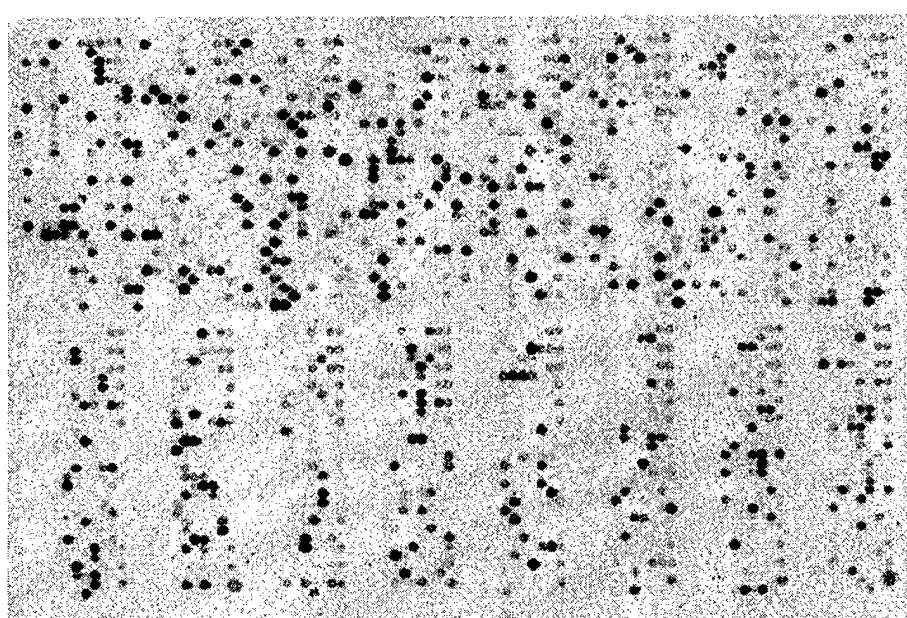
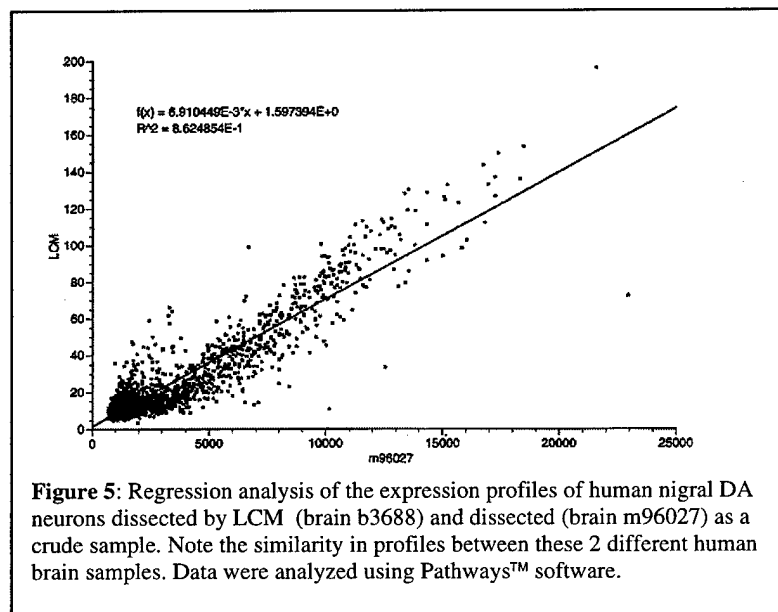


Figure 4: Human "Named Gene" microarray filter (Research Genetics GF-211) hybridized with a ^{33}P -labelled DNA pool generated from LCM-dissected human neurons.

Atlas Human Neurobiology cDNA arrays (Clontech cat. # 7736-1) contain 588 known cDNAs. These neuro-specific custom arrays are ideal for our LCM studies as they can be screened using relatively small amounts (0.5-1 ug) of total RNA as probe template. These filters have similarly been screened with a ^{33}P -labelled DNA pool generated from dissected neurons from a post-mortem human brain (m96027). The hybridized membrane was opposed to a phosphor-imager screen for 48 hrs and the resulting image imported and analyzed using Research Genetics software, PathwaysTM. As with the Research Genetics array, a restricted pattern of mRNA expression was observed. To increase the sensitivity of our technique using Atlas Arrays we are currently improving our protocol.

Regression analysis:

To verify the application of LCM using fresh-frozen human post-mortem brain tissue the genetic profiles of nigral DA neurons have been compared using the Human "Named Gene" filters from Research Genetics. For brain b3688, nigral DA neurons were laser dissected (LCM) while for brain m96027 a pigmented region of the SNpc was dissected by hand (27 mg) and both samples were processed for RNA extraction, processed for first strand DNA synthesis, labeled with [³³P]-dATP/[³³P]-CTP (as described in the Specific Methods below) hybridized to GF-211 microarray filters, washed stringently and then opposed phosphorimager screens for 48 hrs. The two data sets were then imported into Pathways™ and the genetic profiles analyzed and compared using regression analysis (**Figure 5**). The correlation co-efficient (r=0.86) suggests that the genetic profiles of the two samples from two different brains are very similar, and that LCM isolation does not alter the pattern of mRNAs obtained.



Measuring expression of neuroprotectant molecules in substantia nigra.

For initial studies, the brains will be chosen from five individuals with no history of neurological disease. All cases will have a post-mortem interval (PMI) of less than 24 hrs. and a brain pH > 6.2. Molecular studies from our group and others have found a tight correlation between brain pH and mRNA integrity. We currently have on hand samples from at least 9 cases meeting these criteria. The genetic profiles of neurons from the dorsal and ventral tiers of SNc, pars lateralis of SN and paranigral nucleus will be dissected separately using LCM. Our preliminary data suggest that sufficient RNA can be obtained from 2000 neurons, a number which can be readily dissected by a single operator in less than one day. Each of the four RNA samples will then be treated with reverse transcriptase and the DNA probe generated using oligo dT and an equimolar ratio of [³³P]-dATP and [³³P]-dCTP to increase the specific activity of the radiolabeled DNA pool and thus increase the maximize the sensitivity of the microarray screen. Each individual radiolabeled probe pool will then be hybridized to a Atlas

Human Neurobiology cDNA array, opposed to phosphorimager plates and the resulting image scanned into ATlasiImage™, for data analysis. All four samples will be processed together in parallel to minimize cross-sample variability. These studies will allow us to determine the molecular profile of gene expression in human dopamine neurons with different vulnerabilities to Parkinson's disease. We will focus in particular on genes known to be involved in oxidative stress.

One of the most important elements of the experimental design is the anatomical validation of differentially expressed mRNAs. This validation process will be carried out on fresh-frozen cryostat sections (12 µm thick) using conventional single and dual-label in situ hybridization. These assays will include the probes for glutathione peroxidase, MNSOD, and bcl-2 described in the proposal, as well as additional genes identified through the array studies.

Subsequent experiments will be carried out using Affymetrix human DNA chips (Hu-6800 set). These human GeneChip arrays allow an extensive complement of human genes (6,800) to be screened in addition to being able to identify genetic polymorphisms. The MGH has recently established a Gene Array Technology Core Facility that provides cDNA probe labeling of the extracted RNA sample, hybridization to Affymetrix human chip sets, scanning and data output. This microarray core facility is run by Dr. Richard Pratt and we intend to take full advantage of this excellent service.

7. KEY RESEARCH ACCOMPLISHMENTS

- a. The finding of increased lipid peroxidation in the subthalamic nucleus in Progressive Supranuclear Palsy.
- b. The development of a novel and accurate measurement of 8-hydroxy-2-deoxyguanosine in human body fluids.
- c. The demonstration that mice overexpressing manganese SOD are protected from MPTP.
- d. The finding that mice overexpressing Bcl2 are protected from MPTP.
- e. The finding that mice with a dominant negative inhibitor of interleukin converting enzyme are protected against MPTP.
- f. The finding that novel free radical spintraps exert neuroprotective effects against MPTP and its oxidative damage.
- g. The finding that supplementation with creatine and cyclocreatine can significantly attenuate MPTP induced depletion of dopamine and loss of tyrosine hydroxylase neurons.

8. REPORTABLE OUTCOMES

- Albers DS, Augood SJ, Martin DM, Standaert DG, Vonsattel JP, Beal MF. Evidence for oxidative stress in the subthalamic nucleus in progressive supranuclear palsy. *J. Neurochem.* 1999; 73: 881-884.
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9. CONCLUSIONS

We have made substantial progress in finding that there are increased markers of oxidative damage in postmortem in a parkinsonian syndrome. We have also developed a unique highly sensitive assay for measurement of oxidative damage to DNA for examination of parkinsonian patients.

Lastly, we have found that a number of novel therapeutic or genetic manipulations can markedly attenuate both oxidative damage and dopaminergic neurotoxicity in the MPTP model of parkinsonism.

These findings really strengthen the implication of oxidative damage in Parkinson's Disease pathogenesis.

10. REFERENCES

None.

11. APPENDICES

1 copy of each of the cited papers in reportable outcomes.

HARVARD MEDICAL SCHOOL

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April 5, 2000

US. Army Medical Research and Materiel Command
ATTN: MCMR-RMI-S
504 Scott ST.
Fort Detrick, Maryland 21702-5012

RE: DAMD-98-1-8619

Dear Sir or Madam,;

I am writing to reiterate my enthusiasm for continued participation in Dr. Beal's project, "Oxidative Damage in Parkinson's Disease." Although Dr. Beal is now located at Cornell University, we would like to establish a subcontract arrangement which would allow us to continue to collaborate in these important studies. In particular, I and my colleagues would like to assist Dr. Beal in the conduct of the anatomical studies described in the original proposal, and in the application of the new technologies of Laser Capture Microdissection and Gene Array Profiling. I believe that the team at Massachusetts General Hospital is uniquely qualified to bring these powerful technologies to bear on the mechanisms of oxidative injury in Parkinson's disease.

Sincerely,

A handwritten signature in cursive script that reads 'Anne B. Young'.

Anne B. Young, M.D., Ph.D.

Principal Investigator/Program Director (Last, first, middle):**BIOGRAPHICAL SKETCH**

Give the following information for the key personnel, consultants, and collaborators listed on page 4.
Photocopy this page for each person.

NAME		POSITION TITLE		
Anne B. Young, MD, PhD		Professor of Neurology		
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)				
INSTITUTION AND LOCATION		DEGREE	YEAR CONFERRED	FIELD OF STUDY
Vassar College, Poughkeepsie, NY		A.B.	1969	Chemistry
Johns Hopkins University, Baltimore, MD		M.D.	1973	Medicine
Johns Hopkins University, Baltimore, MD		Ph.D.	1974	Pharmacology
Mt. Zion Hosp. & Med Ctr, San Francisco, CA		-	1975	Medicine Internship
U. California San Francisco, Dept. of Neurology		-	1978	Neurology Resident

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government Public Advisory Committee. List, in chronological order, the titles and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

Professional Positions:

- 1978-1982 Assistant Professor, Department of Neurology, University of Michigan
1982-1985 Associate Professor, Department of Neurology, University of Michigan
1985-1991 Professor, Department of Neurology, University of Michigan
1989-1993 NINDS Neurological Disorders Program Project Review B Committee
1991- Julieanne Dorn Professor of Neurology, Harvard Medical School
1991- Chief, Neurology Service, Massachusetts General Hospital
1994-1997 National Advisory Council on Aging, National Institutes of Health

Awards and Other Professional Activities:

- 1969 Phi Beta Kappa
1973 Fellowship from the Scottish Rite Foundation, 33°, Lexington, MA
1979-1984 Teacher-Investigator Development Award, National Institutes of Health
1987-1989 Merck Faculty Development Award
1989 Merck Wexler Award for Huntington's Disease Research, HDSA
1990 Weinstein-Goldenson Award, United Cerebral Palsy Associations, Inc.
1994- Member, Institute of Medicine
1995- Fellow, American Academy of Arts and Sciences
1996 David Seegal AOA Visiting Professorship, Columbia University, NY, NY
1996 Presidential Lecturer, American Academy of Neurology
1997 Anita Harding Memorial Lecturer, Queen's Square Hospital, London
1997 Guthrie Family Humanitarian Award
1997 Royal College Lecturer, Canada
1999 Dean's Award for Support and Advancement of Women Faculty, Harvard Med. School
1999 Anita Harding Memorial Lecture, European Neurological Society, Milan, Italy
1999 Soriano Award, American Neurological Association

Publications: (Partial Listing)

- Sapp E, GE P, Aizawa H, Bird E, Penney J, Young AB, Vonsattel JP, DiFiglia M: Evidence for a preferential loss of enkephalin immunoreactivity in the external globus pallidus in low grade Huntington's disease using high resolution image analysis. *Neurosci* 64:397-404, 1995.
Aronin N, Chase K, Young C, Sapp E, Schwarz C, Matta N, Kornreich R, Landwehrmeyer B, Bird E, Beal MF, Vonsattel JP, Smith T, Caraway R, Boyce F, Young A, Penney J, DiFiglia M: CAG expansion affects the expression of mutant huntingtin in the Huntington's Disease brain. *Neuron* 15:1-20, 1995.
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Rudolf GD, Cronin CA, Landwehrmeyer GB, Standaert DG, Penney JB Jr, Young AB: Expression of NMDA glutamate receptor subunits in the prefrontal cortex of the rat. *Neurosci* 73:417-427, 1996.

Principal Investigator/Program Director (Last, first, middle):

- Standaert D.G., Landwehrmeyer GB, Kerner J, Penney JB Jr, Young AB: Expression of NMDAR2D glutamate receptor subunit mRNA in neurochemically identified interneurons in the rat neostriatum, neocortex and hippocampus. *Mol Brain Research* 42:89-102, 1996.
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Principal Investigator/Program Director (*Last, first, middle*):

- Standaert DG, Friberg IK, Landerhormeyer GB, Young AB, Penney JB Jr. Expression of NMDA glutamate receptor subunit mRNAs in neurochemically identified projection and interneurons in the striatum of the rat. *Brain Res Mol Brain Res*, 64 (1):11-23, 1999.
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BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person.

NAME		POSITION TITLE	
David G. Standaert, M.D., Ph.D.		Assistant Professor of Neurology	
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Harvard University, Cambridge, MA	A.B.	1982	Biochemistry
Washington University, St. Louis, MO	M.D., Ph.D.	1988	Medicine, Pharmacology
Jewish Hospital, St. Louis, MO			Internal Medicine
University of Pennsylvania, Philadelphia, PA			Neurology

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceed two pages, select the most pertinent publications. DO NOT EXCEED TWO PAGES.

Professional Positions:

7/92-6/95 Research and Clinical Fellow, Department of Neurology, Massachusetts General Hospital, Boston, MA
7/95-present Assistant Professor of Neurology, Harvard Medical School, Boston, MA
7/95-present Assistant Neurologist, Massachusetts General Hospital, Boston, MA

Award and Other Professional Activities:

1982 Graduated magna cum laude from Harvard University
1988 Irwin Levy Prize in Neurology and Neurological Surgery
1991 Sam Zeritsky Resident's Research Award in Neurology
1992-1995 American Academy of Neurology Research Fellowship Award in Neuropharmacology
1992-1995 Howard Hughes Medical Institute Postdoctoral Research Fellowship for Physicians
1994, 1995 National Parkinson Foundation Research Award
1996 Cotzias Fellowship, American Parkinson's Disease Association

Publications: (Partial Listing)

Wong SS, JA DiMicco, DG Standaert, KL Dretchen (1977) Beneficial effect of fluorocarbon emulsion media on the function of neuromuscular preparations *in vitro*. J Gen Physiol 69:655-666.
Schwartz TL, GMH Lee, KK Siwicki, DG Standaert, EA Kravitz (1984) Proctolin in the lobster: the distribution, release, and chemical characterization of a likely neurohormone. J Neurosci 4:1300-1311.
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- Weiss SW, DS Albers, MJ Iadorola, TM Dawson, VL Dawson, DG Standaert (1998) NMDAR1 glutamate receptor subunit isoforms in neostriatal, neocortical, and hippocampal nitric oxide synthase neurons. *J Neurosci* 18:1725-1734.
- Albers DS, SJ Augood, DM Martin, DG Standaert, JP Vonsattel, MF Beal (1999) Evidence for oxidative stress in the subthalamic nucleus in progressive supranuclear palsy. *J Neurochem* 73:881-884.
- Albers DS, SW Weiss, MJ Iadorola, DG Standaert (1999) Immunohistochemical localization of NMDA and AMPA receptor subunits in the substantia nigra pars compacta of the rat. *Neuroscience* 89:209-220.
- Augood SJ, DM Martin, LJ Ozelius, XO Breakfield, JB Penney, DG Standaert (1999) Distribution of the mRNAs encoding torsinA and torsinB in the normal adult human brain. *Ann Neurol* 46:761-769.
- Bradley SR, DG Standaert, AI Levey, PJ Conn (1999) Distribution of group III mGluRs in rat basal ganglia with subtype-specific antibodies. *Ann NY Acad Sci* 868:531-534.
- Bradley SR, DG Standaert, K Rhodes, H Rees, C Testa, A Levey, P Conn (1999) Immunohistochemical localization of subtype 4a metabotropic glutamate receptors in the rat and mouse basal ganglia. *J Comp Neurol* 407:33-46.
- Dunah AW, Y Wang, RP Yasuda, K Kameyama, RL Haganir, BB Wolfe, DG Standaert (1999) Alterations in subunit expression, composition and phosphorylation of striatal NMDA glutamate receptors in the rat 6-OHDA model of Parkinson's disease. *Mol Pharmacol*, in press.
- Kosinski CM, SR Bradley PJ Conn, AI Levey, GB Landwehrmeyer, JB Penney, AB Young, DG Standaert (1999) Localization of metabotropic glutamate receptor 7 mRNA/7a protein in the rat basal ganglia. *J Comp Neurol* 415:266-284.
- Kuppenbender KD, DG Standaert, TJ Feuerstein, JB Penney, AB Young, GB Landwehrmeyer (1999) Expression of NMDA receptor subunit mRNAs in neurochemically identified projection and interneurons in the human striatum. *J Comp Neurol*, in Press.

- Kuppenbender KD, DS Albers, MJ Iadarola, GB Landwehrmeyer, DG Standaert (1999) Localization of alternatively spliced NMDAR1 glutamate receptor isoforms in rat striatal neurons. *J Comp Neurol* 415:204-217.
- Simon DK, DG Standaert (1999) Neuroprotective therapies. *Med Clin North Am* 83:509-523.
- Standaert DG (1999) NMDA receptors and nitric oxide synthase. *Mol Psychiatry* 4:13-14.
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- Albers DS, SJ Augood, LC Park, SE Browne, DM Martin, J Adamson, M Hutton, DG Standaert, JP Vonsattel (2000) Frontal lobe dysfunction in progressive supranuclear palsy: Evidence for oxidative stress and mitochondrial impairment. *J Neurochem* 74:878-881.
- Dunah AW, Y Wang, RP Yasuda, K Kameyama, RL Haganir, BB Wolfe, DG Standaert (2000) Alterations in subunit expression, composition, and phosphorylation of striatal N-methyl-D-aspartate glutamate receptors in a rat 6-hydroxydopamine model of Parkinson's disease. *Mol Pharmacol* 57:342-352.

Principal Investigator/Program Director (Last, first, middle):

BIOGRAPHICAL SKETCH

Give the following information for the key personnel, consultants, and collaborators listed on page 4.
Photocopy this page for each person.

NAME		POSITION TITLE		
Sarah Jane Augood, Ph.D.		Instructor in Neurology		
EDUCATION (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)				
INSTITUTION AND LOCATION		DEGREE	YEAR CONFERRED	FIELD OF STUDY
University of East Anglia, Norwich, UK		B.Sc.	1984	Chemistry
Council for National Academic Awards, London, UK		Ph.D.	1992	Neuroanatomy and Neurobiology

RESEARCH AND/OR PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order previous employment, experience, and honors. Key personnel include the principal investigator and any other individuals who participate in the scientific development or execution of the project. Key personnel typically will include all individuals with doctoral or other professional degrees, but in some projects will include individuals at the masters or baccalaureate level provided they contribute in a substantive way to the scientific development or execution of the project. Include present membership on any Federal Government Public Advisory Committee. List, in chronological order, the titles and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. DO NOT EXCEED TWO PAGES.

Professional Positions:

1991-1993 Postdoctoral Fellow, Department of Psychiatry, University of Cambridge, UK
1993-1994 Postdoctoral Fellow, Department of Anatomy, University of Cambridge, UK
1994-1997 Wellcome Trust Mental Health Research Fellow, Neurobiology, Babraham Institute, Cambridge, UK
1997-1998 Visiting Research Fellow in Neurology, Neurology Service, Massachusetts General Hospital, Boston, MA
1999-present Instructor in Neurology, Neurology Service, Massachusetts General Hospital, Boston, MA
1999-present Instructor in Neurology, Harvard Medical School, Boston, MA

Awards and Other Professional Activities:

1988 Guarantors of Brain Travel Award, 9th International Symposium on Parkinson's Disease, Israel
1991 Biochemical Society Travel Award, 21st Annual Meeting of the Society of Neuroscience, USA
1991 Tourette Syndrome Association (USA) Research Award
1992 Royal Society Equipment Award
1992 SmithKline (1982) Foundation Consumable Award
1993 European Science Foundation Travel Award
1993 Wellcome Trust Travel Award
1994 Wellcome Trust Mental Health Training Fellowship
1997 Parkinson's Disease Society Research Award

Publications:

Augood SJ, Emson PC, Mitchell IJ, Boyce S, Clarke CE, Crossman AR. (1989) Cellular localization of enkephalin gene expression in MPTP-treated cynomolgus monkeys. *Mol. Brain Res.*, 6, 85-92.
Davenport AP, Augood SJ, Lawson DEM, Emson PC. (1990) The use of quantitative immuno-cytochemistry (QICC) to measure calbindin D-28k like immunoreactivity in the rat brain. *Cell. Mol. Biol.* 36, 1-11.
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Give the following information for the key personnel, consultants, and collaborators listed on page 4.
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Rapid Communication

Evidence for Oxidative Stress in the Subthalamic Nucleus in Progressive Supranuclear Palsy

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Abstract: Increased free radical production and oxidative stress have been proposed as pathogenic mechanisms in several neurodegenerative disorders. Free radicals interact with biological macromolecules, such as lipids, which can lead to lipid peroxidation. A well-established marker of oxidative damage to lipids is malondialdehyde (MDA). We measured tissue MDA levels in the subthalamic nucleus (STN) and cerebellum from 11 progressive supranuclear palsy (PSP) cases and 11 age-matched control cases using sensitive HPLC techniques. In PSP, a significant increase in tissue MDA levels was observed in the STN when compared with the age-matched control group. By contrast, no significant difference between tissue MDA content was observed in cerebellar tissue from the same PSP and age-matched control cases. These results indicate that lipid peroxidation may play a role in the pathogenesis of PSP. **Key Words:** Neurodegenerative disorders—Free radicals—Lipid peroxidation—Malondialdehyde—Subthalamic nucleus—Progressive supranuclear palsy.

J. Neurochem. **73**, 881–884 (1999).

Progressive supranuclear palsy (PSP) is a rare neurological disorder associated with impairments of gait, balance, and cognition. The clinical symptoms of PSP include supranuclear ophthalmoplegia, postural instability, dysarthria, truncal dystonia, parkinsonism, and dementia. PSP was first described as a discrete clinicopathological entity in 1964 (Steele et al., 1964), although an isolated case had been reported by Chavany and colleagues in 1951 (Chavany et al., 1951). In the early stages of the disease, PSP can be misdiagnosed as Parkinson's disease (PD), particularly if the supranuclear ophthalmoplegia is absent. Pathologically, PSP is characterized by extensive neuronal degeneration in cortical and subcortical nuclei and by the presence of neurofibrillary and tau-positive tangles; the subthalamic nucleus (STN) is one of the most severely affected structures (Steele et al., 1964; Hauw et al., 1994; Litvan et al., 1996).

The STN is a bilateral elliptical structure located between the thalamus and midbrain. It is composed primarily of tonically active glutamatergic projection neurons (Albin et al., 1989; Rinvik and Ottersen, 1993). Overactivity of these neurons is now known to contribute to the clinical expression of parkinsonism (Mitchell et al., 1989a), as symptoms can be

alleviated by surgical or pharmacological intervention in this nucleus (Bergman et al., 1990; Benazzouz et al., 1993).

The etiological basis of PSP is unknown, although recent genetic studies have identified polymorphisms at the *tau* locus as a potential risk factor (Conrad et al., 1997; Baker et al., 1999). Mechanistically, compelling data suggest that "oxidative stress" contributes to the pathogenesis of several other neurodegenerative disorders, including Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and PD (for review, see Beal, 1997). In particular, increased tissue malondialdehyde (MDA) levels and lipid hydroperoxides were found in the parkinsonian substantia nigra, providing the first direct biochemical evidence of oxidative mechanisms of cell injury in PD (Dexter et al., 1989). Increases in tissue MDA levels have been reported in postmortem tissue from Alzheimer's disease and amyotrophic lateral sclerosis—other neurodegenerative diseases associated with increased oxidative damage (for review, see Beal, 1997). Thus, we undertook this study to search for evidence of oxidative stress in PSP, by determining tissue MDA levels in the STN, one of the brain structures most severely affected by the degenerative process of PSP (Steele et al., 1964; Hauw et al., 1994; Litvan et al., 1996).

MATERIALS AND METHODS

Human brain tissue

Tissue from 11 pathologically confirmed cases of PSP [age range, 60–87 years; postmortem interval (PMI) range, 4.0–31.2 h] and 11 control cases (age range, 51–91 years; PMI range, 8.7–32.5 h) was provided by the Harvard Brain Tissue Resource Center (Belmont, MA, U.S.A.). The pathological diagnosis was made by examination of the contralateral hemisphere using the National Institute of Neurological Disorders and Stroke pathological criteria for PSP (Hauw et al., 1994;

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Abbreviations used: MDA, malondialdehyde; PD, Parkinson's disease; PMI, postmortem interval; PSP, progressive supranuclear palsy; STN, subthalamic nucleus.

TABLE 1. PSP and control cases used in the present study

Case	Cause of death	Diagnosis	Age (years), sex	PMI (h)
B3972	Congestive heart failure	PSP	73, M	4.75
B4044	Unknown	PSP	75, F	8.6
B3973	Pneumonia	PSP	87, F	11.25
B3938	Heart attack	PSP	81, F	21.5
B4027	Unknown	PSP	69, M	4.0
B4016	Pneumonia	PSP	81, F	5.75
B3926	Myocardial infarction	PSP	74, M	13.4
B3857	Pneumonia	PSP	74, F	18.4
B3727	Heart attack	PSP	78, F	8.0
B3549	Heart attack	PSP	60, M	31.2
B4208	Congestive heart failure	PSP	77, M	17.3
B3296	Unknown	Control	76, F	32.5
B3896	Cancer	Control	84, F	11.85
B3729	Coronary artery disease	Control	91, F	15.4
B3746	Unknown	Control	83, M	25.25
B3941	Cancer	Control	63, M	23.0
B3979	Heart attack	Control	62, M	13.2
B3983	Heart attack	Control	71, M	8.7
B4019	Lung cancer	Control	65, M	16.2
B4030	Heart attack	Control	81, F	11.5
B4034	Cardiac-related	Control	88, M	16.25
B4077	Myocardial infarction	Control	51, M	10.2

Litvan et al., 1996). The 11 control cases had a distributive diagnosis of control with no history of neurological disease (Table 1). Of the 11 PSP cases, cerebellar cortex tissue was available in all cases, whereas STN tissue was available in only eight of the cases. Of the 11 control cases, cerebellar cortex tissue was available from all brains, whereas STN tissue was available in only 10 cases. Fresh frozen tissue was received either in a vial with the STN or cerebellar cortex already dissected or as a frozen tissue block ($\sim 5 \times 5 \times 2$ cm), in which case the required structure was dissected at Massachusetts General Hospital. To verify accurate dissection of the STN from thalamic blocks, cryostat sections were cut (12 μ m) and counterstained with methylene blue. All tissue samples were dissected, stored at -80°C , and processed for MDA determination in parallel.

HPLC determination of tissue MDA levels

Thiobarbituric acid-reactive substances were prepared from blinded samples and assayed using sensitive HPLC with fluorometric detection as described previously (Halliwell and Chirico, 1993; Ferrante et al., 1997). In brief, tissue was sonicated (1:50 wt/vol) in 100 mM potassium dihydrogen phosphate (pH 7.4)/butylated hydroxytoluene before mixing aliquots of each sample with an equal volume of thiobarbituric acid in sodium acetate buffer. Samples were incubated at 93°C for 45 min and then diluted (1:1 vol/vol) with butanol/pyridine (15:1 vol/vol) before being injected on an HPLC [Microsorb 5- μ m (pore size) C18 analytical column] coupled with a Waters model 470 fluorescence detector ($\lambda_{\text{ex}} = 530$ nm; $\lambda_{\text{em}} = 552$ nm). The mobile phase consisted of 40% methanol and 60% 50 mM potassium dihydrogen phosphate (pH 7.0) filtered and degassed before use, with a flow rate of 0.9 ml/min. The retention time for the single resulting fluorescent peak under the chromatographic conditions used was 6.6 min, comigrating with authentic MDA. Quantitation of MDA levels was based on

integration of peak area and compared with MDA standards. Data are expressed in micromoles of MDA per milliliter.

Statistics

Tissue MDA levels for the PSP and control groups are presented as mean \pm SEM values. Statistical comparisons between PSP and control values were made using the Mann-Whitney *U* test (InStat; GraphPad, San Diego, CA, U.S.A.).

RESULTS AND DISCUSSION

There was no significant difference in age or PMI between the PSP and control groups. Tissue MDA levels in the STN and cerebellum in PSP and control cases are presented in Fig. 1. In the STN, a significant increase (275%) in tissue MDA levels was observed in the PSP group (0.55 ± 0.06 $\mu\text{mol/ml}$) as compared with the control group (0.20 ± 0.01 $\mu\text{mol/ml}$). No correlation was observed with age or PMI. In contrast, tissue MDA levels in the PSP cerebellar cortex were similar to levels observed in control tissue (control, 0.33 ± 0.02 $\mu\text{mol/ml}$; PSP, 0.38 ± 0.03 $\mu\text{mol/ml}$). These data provide evidence to suggest that oxidative damage contributes to the pathogenesis of PSP.

The increased MDA levels in the STN are of particular interest as they underline the importance of this brain region in the expression of aberrant movement disorders (Mitchell et al., 1989a,b; Beurrier et al., 1997). Furthermore, the absence of an increase in the PSP cerebellum from the same cases demonstrates that oxidative mechanisms of injury are regionally specific (Steele et al., 1964; Hauw et al., 1994; Litvan et al., 1996). Future studies are required to investigate if tissue MDA levels are increased in other degenerating brain areas in PSP.

Substantial evidence has implicated oxidative stress in several other neurological and neurodegenerative diseases, including PD, stroke, Huntington's disease, amyotrophic lateral sclerosis, and Alzheimer's disease (for review, see Beal, 1997). In PD, increased levels of MDA and lipid peroxides have been reported in the substantia nigra (Dexter et al., 1989), the site of primary pathology. Furthermore, oxidative damage to DNA in the PD striatum and substantia nigra has been reported (Sanchez-Ramos et al., 1994) in addition to decreased glutathione levels (Dexter et al., 1994) and increased iron levels (Dexter et al., 1987). Our findings therefore add PSP to the list of neurodegenerative disorders involving neuronal oxidative stress.

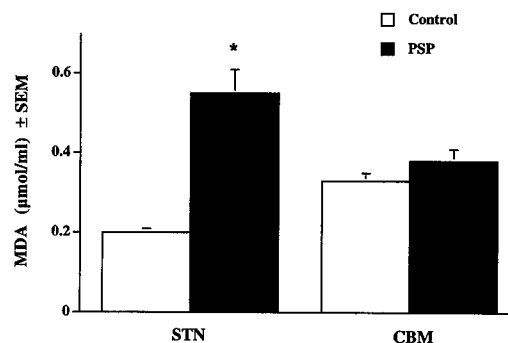


FIG. 1. Tissue MDA levels in STN and cerebellum (CBM) from PSP ($n = 8$, STN; $n = 11$, CBM) and control ($n = 10$, STN; $n = 11$, CBM) cases. Data are mean \pm SEM (bars) values, given as $\mu\text{mol/ml}$. * $p < 0.001$, statistically significantly different from control STN.

Thus, the critical question is now whether oxidative injury plays a causative role in the pathogenesis of PSP or is merely a secondary phenomenon. The human STN may be particularly sensitive to oxidative damage because it receives a major glutamatergic input from the cerebral cortex and parafascicular nucleus (for review, see Joel and Weiner, 1997), contains NMDA receptors (Ball et al., 1994), and is enriched in neuronal nitric oxide synthase (Eve et al., 1998). Excessive stimulation of glutamate receptors, particularly NMDA receptors, has been associated with generation of reactive oxygen species. In addition, nitric oxide synthase, which is stimulated by NMDA receptor activation, generates nitric oxide and other reactive oxygen species. The combined effect of nitric oxide synthase and NMDA receptor activation, i.e., excitotoxicity, can lead to oxidative injury and cell death. In other neurodegenerative diseases, oxidative injury has also been attributed to defects in specific mitochondrial complex activities (for review, see Beal, 1997). For example, the reduction in complex I activity in PD results in mitochondrial dysfunction increasing the vulnerability of nigral dopaminergic neurons to glutamate. In PSP, evidence for metabolic impairment has been shown in patients using positron emission tomography as well as in muscle biopsy specimens (Blin et al., 1990; Di Monte et al., 1994). Therefore, it is tempting to speculate that in the event of some primary defect, e.g., mitochondrial dysfunction, NMDA receptor overactivation and/or excessive production of nitric oxide could lead to cell death via oxidative mechanisms and that the STN may be particularly vulnerable to such injury.

In summary, our findings indicate that oxidative mechanisms of injury are involved in the pathogenesis of PSP. Thus, future research into the development of new and improved antioxidants may hold great promise as a therapy that could slow or halt the neurodegenerative progression of this debilitating disorder, as has been shown in Huntington's disease and an animal model of familial amyotrophic lateral sclerosis (Koroshetz et al., 1997; Klivenyl et al., 1999).

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 **Original Contribution**

A CARBON COLUMN-BASED LIQUID CHROMATOGRAPHY ELECTROCHEMICAL APPROACH TO ROUTINE 8-HYDROXY-2'- DEOXYGUANOSINE MEASUREMENTS IN URINE AND OTHER BIOLOGIC MATRICES: A ONE-YEAR EVALUATION OF METHODS

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Abstract—8-Hydroxy-2'-deoxyguanosine (8OH2'dG) is a principal stable marker of hydroxyl radical damage to DNA. It has been related to a wide variety of disorders and environmental insults, and has been proposed as a useful systematic marker of oxidative stress. Analytic procedures for 8OH2'dG in DNA digests are well established; however, routine measurement of free 8OH2'dG in other body fluids such as urine or plasma has been problematic. This has hindered its evaluation as a general clinical, therapeutic monitoring, or environmental assessment tool. Therefore, we developed a liquid chromatography electrochemical column-switching system based on the use of the unique purine selectivity of porous carbon columns that allows routine accurate measurement of 8OH2'dG in a variety of biologic matrices. This paper describes the rationale of the system design and the protocols developed for 8OH2'dG in urine, plasma, cerebrospinal fluid, tissue, DNA, saliva, sweat, kidney dialysis fluid, foods, feces, culture matrix, and microdialysates. Concentrations in both human and animal body fluids and tissues are reported. The system performance is discussed in the context of a 1-year evaluation of the methods applied to approximately 3600 samples, using internal quality control and external blind testing to determine long-term accuracy. The methods are reliable and accurate, and therefore should prove useful in assessing the role and utility of oxidative DNA damage in aging and human illness. © 1999 Elsevier Science Inc.

Keywords—8-Hydroxy-2'-deoxyguanosine, Carbon columns, Chromatography, High-pressure liquid, Urine, Plasma, Cerebrospinal fluid, DNA, Free radicals

INTRODUCTION

The concentrations of products from oxidatively damaged macromolecules may serve as indicators of oxidative stress. Several different DNA-base adducts were identified after exposure of mammalian chromatin to ionizing radiation-generated free radicals [1]. Of these, 8-hydroxy-2'-deoxyguanosine (8OH2'dG) is the most common oxidative DNA lesion [2]. Increases in 8OH2'dG levels have been implicated in a number of disorders, including cancer [3,4], neurodegenerative dis-

eases [5–7], and diabetes [8,9]. Increases have been seen in aging [3,4,10], exposure to different toxic agents, and environmental insults [11–15]. Concentrations increase with exposure to oxidants, both in experimental animals and humans [16]. Increased urinary levels were reported in patients with cystic fibrosis, normal levels in patients with hemochromatosis, and decreased levels in patients with systematic lupus erythematosus [17–19]. Studies have also shown the lack of correlation of urinary 8OH2'dG with the antioxidants vitamin E, vitamin C, and coenzyme Q10 supplements [20], and the lack of effect of these materials on urinary 8OH2'dG levels in smokers [21]. Overall, the approximately 450 citations of the last decade suggest that 8OH2'dG is a useful biologic marker for assessment of oxidative damage. The effec-

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tive use of this body of knowledge for elucidating disorder mechanisms, assessing risk, or monitoring therapy has, however, been hindered by a lack of routine high-throughput analytic methodology.

The analysis of 8OH2'dG in DNA digests is well established, although there is considerable debate about isolation and preparative protocols [22]. However, assays of free levels in other tissues have been problematic. The routine measurement of 8OH2'dG in biologic samples, and particularly in urine, is inherently a challenging analytic problem because of the low levels of the analyte and the complexity of the samples. This problem is confounded by a high level of variability of potential interferences in different samples. A variety of techniques have been introduced to measure 8OH2'dG, including gas chromatography-mass spectroscopy (GCMS) [18,23], enzyme-linked immunosorbant assay (ELISA) [24,25], liquid chromatography-mass spectrometry (LCMS) [26], and liquid chromatography with electrochemical detection (LCEC) [13,27-29]. Additionally, LCEC methods have used column-switching approaches and/or sample preparation with various solid phase extraction (SPE) columns to achieve selectivity [12,29-34].

The requirements for an 8OH2'dG assay are applicability to a range of biologic samples in a variety of different disorders, rapid throughput, high precision, and long-term accuracy. The application to multiple tissues is essential for the determination of the compartmental distribution and biokinetics of 8OH2'dG. Application to a range of disorders, high throughput, and precision are needed for evaluation of small categorical differences and long-term changes with intervention in patient cohorts. Applicability to a variety of disorders is particularly important because of interferences and complexity introduced by diseases themselves and associated pharmacologic therapies.

Previously reported procedures of preparative isolation using SPE columns, in our hands, required a level of manipulation that was incompatible with high sample throughput. We evaluated a number of protocols utilizing SPE columns (C18, C8, anion, cation, and immunoaffinity) followed by a gradient LCEC system with multiple coulometric electrodes to assess peak purity. Solid phase extraction protocols substantially reduced the complexity of the chromatograms for 8OH2'dG in a gradient coulometric array system. However, 8-14 possible interference peaks were still found in urine preparations, and these varied significantly among individuals and disorders. The major difficulty was the frequency with which minimally resolved coeluting peaks and highly inaccurate ratios of coulometric response for 8OH2'dG were found in individual samples. Coulometric electrodes in series set at sequentially increasing potentials across the

low-to-high response region of the current vs. potential curve of any analyte give a current response at each electrode that is characteristic of the analyte. Deviation from the ratio of these responses is indicative of a coeluting contaminant. Although gradient conditions could be varied in most cases to resolve the 8OH2'dG signal, it was not possible to find a set of conditions general for all samples investigated [29].

The previously described column-switching LCEC method using a cation exchange column to trap the 8OH2'dG eluting from the first column has been well documented and used to produce a large body of data on urinary 8OH2'dG levels in normal individuals [13,20]. With this approach, we found equivalent precision and no incidence of interference for control individuals. However, we encountered significant problems with the majority of urine samples from patients with Parkinson's disease (PD), and Alzheimer's disease (AD), and amyotrophic lateral sclerosis (ALS), and from children with cerebral palsy (CP). For most samples, the interferences could be resolved by variation of chromatographic or timing conditions, or by combined use of SPE protocols. However, no single protocol was found that was applicable to all samples.

Because of its demonstrated utility and capacity for automation, the column-switching protocol is an attractive approach to the analytic problem. However, the recurrent problem of individual specific and disorder-related interferences indicates a fundamental difficulty in making it general for all sample types. Essentially, all SPE and column-switching separation procedures rely on variations of polymer and silica packing materials. These are not vastly different in retention and elution characteristics for 8OH2'dG and the range of possible unknown interferences. These observations suggested an approach based on different trapping column materials with characteristics highly dissimilar to those of silica and polymer-based materials. We found that classes of carbon materials could be treated to create unique selectivity to purines. Consequently, we initiated a series of investigations to incorporate treated porous carbon columns into an LCEC column-switching analytic system following the basic strategy previously developed for 8OH2'dG assays [13,20].

The overall objective of the work was 3-fold: first, to evaluate the suitability of the carbon column system for utilizing urinary 8OH2'dG measurements in long-term longitudinal studies; second, to provide assay methods for determining compartmental relationships among urine, plasma, red blood cells, cerebrospinal fluid (CSF), saliva, sweat, and the effects of these compartments on the utility of urinary measurements; and third, to provide methods for cell culture matrices, food, feces, and dialysis samples for investigation of issues such as the con-

tribution of apoptosis, diet, and metabolism, and sources of production of 8OH2'dG.

In the design of the study, system conditions and preparative procedures were standardized and evaluated for 1 year on approximately 3600 samples. Particular effort was made to assess the level of confidence that could be placed in urinary 8OH2'dG data obtained over various time intervals, and to evaluate the range of applicability to different species and disorders. As a corollary to these objectives, issues of artifacts from sample acquisition, storage, and preparation were also investigated.

MATERIALS AND METHODS

Equipment and reagents

Various combinations of electrochemical detectors and sensors, autosamplers, and pumps were used for different aspects of this study (ESA Inc., Chelmsford, MA, USA) as follows: model 5600 16-channel gradient Coullarray system; model 5200A Coulochem II detector with four potentiostats and various coulometric cells (models 5010, 5011, 5014B, and 5021); model 580 pumps; model 460 autosampler; and model 70-114 column heater. Six-port high-pressure valves were used (model EC6W; Valco Instruments Co., Inc., Houston, TX, USA). The low-pressure valve was from Alltech Associates Inc. (Deerfield, IL, USA). Column-switching, system control, and data analysis were performed using an ESA 501 data station. The C8 column was a YMC basic 3 μm , $4.6 \times 150\text{-mm}$ (YMC Inc., Wilmington, NC, USA), and the C18 column was a 5- μm , $4.6 \times 250\text{-mm}$ TSK-GEL ODS-80TM from TosoHaas (Montgomeryville, PA, USA). In-house water was deionized and double distilled. Acetonitrile (Acn), methanol (MeOH), and methylene chloride (MeCl_2) were from EM Science (Gibbstown, NJ, USA). Lithium salts were prepared in-house from lithium hydroxide monohydrate (Aldrich, Milwaukee, WI, USA) and glacial acetic acid (J.T. Baker, Phillipsburg, NJ, USA). 2'-Deoxyguanosine (2'dG), 2'-deoxyguanosine-5-monophosphate (2'dG5MP), calf thymus DNA, adenosine, and other authentic reference materials were from Sigma Inc. (St. Louis, MO, USA). Nuclease P1 was from Calbiochem Inc. (La Jolla, CA, USA). Alkaline phosphatase and trypsin were from Boehringer-Mannheim GmbH (Mannheim, Germany). 8-Hydroxy-2'-deoxyguanosine was from ESA Inc.

Carbon columns were prepared at ESA Inc. based on previously described diffusion modification procedures [35]. The unique properties of carbon columns provided the key element of the analytic system. The columns were made from an integral sintered porous carbon using a single 6-ft³ furnace run of PS-2 grade porous carbon from Poco Graphite Inc. (Decatur, TX, USA), (ESA Inc.,

no. 42-1652) with a pore size of 0.8–0.9 μm and a density of 1.08–1.12 g/cm^3 as a starting material. Used directly, the material had variable chromatographic response. The carbon was treated by oxidative-reductive cycling to provide a high selectivity for purines and nitro-substituted aromatic compounds. Carbon sections from half-cubic-foot billets were selected based on backpressure of 5–7 bar for test pieces of 0.62-cm diameter and 0.62-cm length at 1 ml/min flow rates in MeOH. Precut cylindrical sections of equivalent backpressure were refluxed in MeCl_2 for 24 h in a Soxhlet apparatus, followed by 10 min in air at $350 \pm 5^\circ\text{C}$ and treatment by water vapor at ambient pressure at $300 \pm 5^\circ\text{C}$ for approximately 24 h until the evolution of 15–20 ml/g of CO and H_2 . The air-water vapor heating cycle was repeated on each lot until the backpressure of test pieces was uniform at 4–4.5 bar. The process provides uniformity of pore size by selective etching of the smaller pores and opening of the nonconnected porosity. The water vapor reduction reactions eliminate surface and subsurface epoxide linkages and provide a stable redox potential of –100 to –120 mV vs. Ag/AgCl in 0.1 M NaCl over periods up to 6 months. Cylindrical sections of carbon material were contained in a 1.265-cm outside diameter sleeve of high molecular weight linear polyethylene constrained at its crush pressure of 400–500 bar in a stainless steel tube, which also housed column end fittings inserted into the polyethylene sleeve. The sleeve/carbon column/end fittings assembly was brought to -70°C , inserted into the stainless steel tube, capped, and allowed to expand to the crush point of the polyethylene at room temperature.

For this work, we evaluated 14 carbon columns with diameter of 0.62 cm, and lengths from 0.62 to 7.5 cm. The columns had 400–480 plates/cm, backpressure of 10–12 bar/cm and void volumes of 90–95 $\mu\text{l/cm}$. The chromatographic characteristics of the carbon columns are illustrated in Fig. 1.

Analytic system configuration, components, and conditions

All analytic data reported in this study were obtained on an analytic system with fixed components and conditions controlled by ESA 501 software. The detector was a four-channel model 5200A Coulochem II with guard cell. The detector was modified for 1 mA full-scale range on channel 1 and 2 (T1 and T2). The valve-switching arrangement and LCEC setup is shown in Fig. 2. An analytic cycle was initiated by the autosampler injection. The band containing 8OH2'dG eluted from the C8 column by mobile phase A (MP A) (0.1 M lithium acetate, pH 6.4, MeOH 4%) was transferred to two serially placed 0.62 \times 0.62-cm carbon columns by switching valve 2 for 1.2 min (gate 1) at times from 11 to 12.2 min;

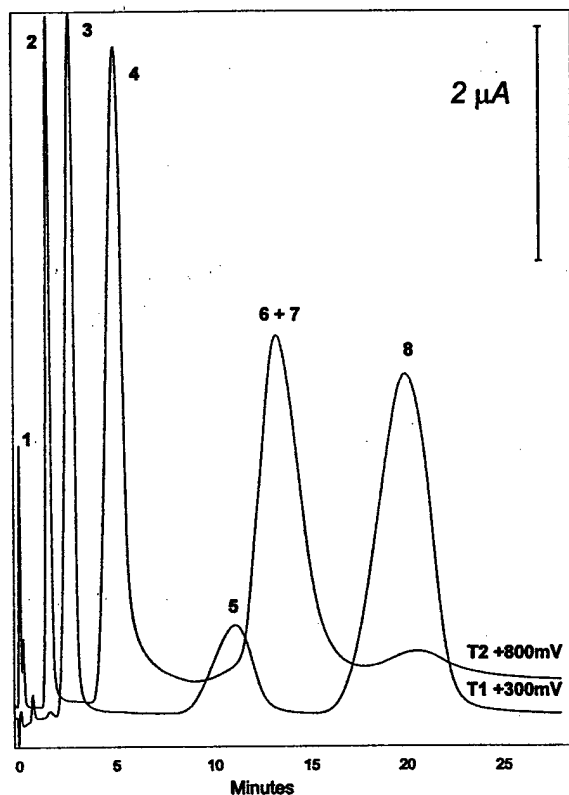


Fig. 1. Typical chromatogram illustrating the unique selectivity of the carbon column to purines. The column dimensions were 0.62×1.25 cm (diameter \times length, void volume = $105 \mu\text{l}$). Mobile phase B (0.1 M lithium acetate, pH 3.3, 4.5% Acn) was delivered at 1 ml/min. T1 and T2 electrodes of a model 5010 cell were set at 300 and 800 mV, respectively. Fifty microliters of standards mixture containing 800 ng/ml of each standard was injected onto the column. Peak 1: tyrosine, tryptophan, and homovanillic acid (coelute in the void of the column); peak 2: 3-chlorotyrosine; peak 3: 5-oxocytidine; peak 4: 3-nitrotyrosine; peak 5: 8-hydroxyguanine; peaks 6 and 7: *o*-6-methylguanine and guanosine (coelute on the column under conditions used); peak 8: 8OH2'dG ($k' = 213$). For comparison, under these conditions on a typical C18 column 8OH2'dG has $k' = 4$.

the C8 column was then backflushed using valve 1 from 13 to 28 min. The high selectivity of the carbon columns retained 8OH2'dG, while potential interferences were eluted and the 8OH2'dG moved quantitatively to the second serial in line carbon column by 9–12 ml of mobile phase B (MP B) (0.1 M lithium acetate, pH 3.3, 4.5% Acn). The second carbon column in the series was then switched to the C18 column using valve 3 for 0.7 min (gate 2) at 17 min with mobile phase D (MP D), which was identical to MP B, but with the addition of adenosine (1.5 g/l) to release the 8OH2'dG. The low-pressure valve then selected mobile phase C (MP C) (MeOH: MeCl₂ 1:1, 0.3 M perchloric acid) from 20 to 27 min to clean both carbon columns. After the backflush of the C8 column was completed, the system initiated the next cycle at 34 min. All columns were maintained at 30°C.

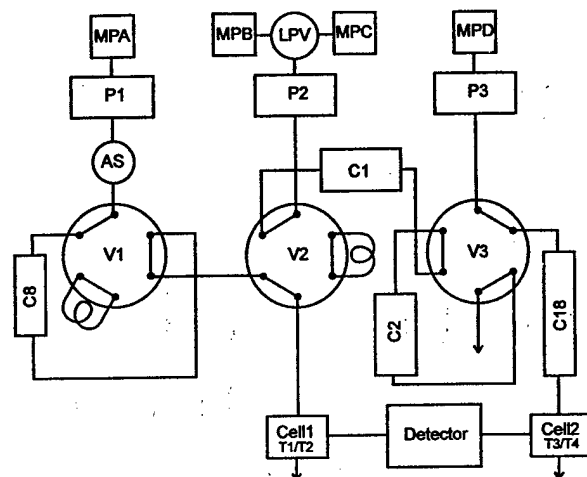


Fig. 2. Liquid chromatography electrochemical setup and valve-switching arrangement of the analytic system for 8OH2'dG (see Materials and Methods section for detailed explanation). P1, P2, and P3 = pumps delivering MP A, MP B or MP C, and MP D, respectively; AS = autosampler; V1–V3 = high-pressure valves 1–3; C8 = C8 column; C18 = C18 column; C1 and C2 = the first and second carbon columns, respectively; LPV = low-pressure valve; cell 1 = model 5010 analytic cell; cell 2 = model 5021 conditioning cell and model 5014B analytic cell in series.

A dual-channel model 5010 cell (sensors T1 and T2) at the output of the C8 column was used to determine the retention time of 8OH2'dG to set the time for valves switching cycles. For DNA digests, the T1 and T2 sensor potentials were set at 820 mV and 840 mV, respectively, to detect 2'dG before switching the C8 column. A model 5021 cell and 5014B cell (sensors T3 and T4) in series were placed at the output of the C18 column to detect 8OH2'dG. The potential of the 5021 cell was set at 40 mV to remove possible low oxidation potential interferences. The T3 and T4 sensors of the 5014B cell were set at 60 mV and 165 mV, respectively, to give a T4/T3 response ratio for 8OH2'dG of approximately 5.

The peak-finding parameters in the ESA 501 software were set for peak retention time intervals of ± 0.2 min, detection thresholds equivalent to a 5 pg/ml standard, and base resolution of 1.5 min equivalent to $2.5\times$ the peak base width. For calculation of values and qualitative measures of peak purity, retention time, peak width at half height ($W_{1/2}$), peak height, and area were reported for T1–T4. 8-Hydroxy-2'-deoxyguanosine values were calculated from the average peak height of bracketing standards. 2'-Deoxyguanosine values were calculated from the average sum of the peak areas of T1 and T2 of bracketing standards. The response ratio of T4/T3, $W_{1/2}$, and retention time were used as qualitative assurance measures for the peak purity of 8OH2'dG.

Variation of analytic system conditions

To determine the final analytic conditions the effects of variations in components and mobile phases were studied. Standards and pools of control, ALS, and CP urines were used as test materials. The composition of MP A was investigated over the range of MeOH 3–8% and pH 4–7; and MP B over the range of 2–8% Acn, and pH 3–7. Adenosine concentrations in MP D were examined over a range of 0.3–2 g/l. Carbon columns with diameter 0.62 cm and lengths from 0.62 to 1.86 cm were investigated in various serial configurations.

Sources of samples

Approximately 1500 urine samples of normal controls (individuals without diagnosed medical disorders) were provided from staff volunteers and family at ESA Inc. and Massachusetts General Hospital (MGH) (Boston, MA, USA), from controls for studies of neurodegenerative disorders at MGH, from RJ Reynolds Tobacco Inc. (Winston-Salem, NC, USA) by Dr. David Doolittle, and for children, from the Institutes for the Achievement of Human Potential (IAHP) (Philadelphia, PA, USA). Approximately 500 urine samples from PD, AD, ALS, Huntington's disease (HD), myopathies, Friedrich's ataxia, and stroke patients were provided from the Movement, Memory, Muscular, Huntington's and Stroke Clinics at MGH. Urine samples from 600 children diagnosed with CP, behavioral disorders, and autism were provided from an existing set of serial urine samples from 1250 cases maintained by the IAHP and ESA Inc. Strips of 150 neonatal urine filter paper samples were provided from St. Marianna Hospital, (Kawasaki, Japan) by Drs. Hitoshi Yamamoto and Kohnosuke Nakata. Approximately 80 samples from individuals with various industrial exposures were provided from the National Institutes for Occupational Safety and Health (NIOSH) (Cincinnati, OH, USA) by Dr. Mark Toraason. Approximately 50 samples of astronaut urine were provided from Johnson Space Center (Houston, TX, USA) by Dr. Jeannie Nillen. Approximately 300 rat urine samples were provided from studies at the Massachusetts College of Pharmacy (MCP) (Boston, MA, USA) by Dr. Timothy Maher. Approximately 80 mouse urine samples from control, HD, and ALS mice were provided from studies at the Department of Neurology at MGH. Approximately 60 monkey urine samples were provided from the University of Wisconsin Primate Center (Madison, WI, USA) by Dr. Richard Weindruch.

Approximately 150 plasma samples from patients with AD, PD, HD, ALS, and stroke were provided from the respective MGH clinics above. Approximately 120 plasma samples from healthy subjects were provided

from in-house volunteers and as controls for studies as above. Twenty plasma samples from kidney dialysis patients were provided from the North County Kidney Center (Fitchburg, MA, USA) by Dr. Francis Zambetti. Thirty rat plasmas were provided from MCP.

Approximately 70 CSF samples for controls, ALS, AD, and PD were provided from MGH clinics, and from a library of disorder and control samples from prior studies maintained at ESA Inc. Saliva and sweat samples were provided from in-house volunteers.

Rat fecal samples were provided from studies at MCP. Rat, mouse, and monkey chow and human foods were obtained from usual commercial sources. Sixty samples of *Caenorhabditis elegans* culture matrix were provided from George Mason University (Fairfield, VA, USA) by Dr. James Willett. Forty samples of kidney dialysis fluid were provided with matched plasmas from the North County Kidney Center. Approximately 150 DNA digests were provided by NIOSH, and an additional 40 calf thymus DNA samples were prepared in-house for analytic evaluation.

All human samples used in this work were obtained under informed consent of the subjects and under protocols approved by the Institutional Review Boards of the relevant organizations for analysis of markers of oxidative stress. All library samples analyzed from previous studies were obtained under protocols allowing analysis of any marker or compound possibly relevant to the disorder studied. The laboratory was blind to the identity of the subjects.

Preparation of standards

Stock standards of 8OH2'dG (100 µg/ml) were made in 0.05% phosphoric acid and kept at +4°C. Working standard solutions were made in mobile phase A and maintained at +4°C. 2'-Deoxyguanosine and 2'dG5MP standards were made in mobile phase A on the day of use.

Evaluation of limits of detection, linearity, precision, and recovery

Linearity and sensitivity were evaluated bimonthly using six repetitive 50-µl injections of 0.01-, 0.03, 0.1-, 0.3-, 1-, 3-, 10-, 30-, and 100-ng/ml standards. Precision was determined bimonthly using repetitive 50-µl injections of a 10-ng/ml standard over 24 h, with the response ratio of T4/T3 set between 4.5 to 5.5.

System recovery was tested by using a model 5010 cell in both cell 1 and cell 2 positions, with all potentials set at 400 mV to measure total coulombs recovered for 1 and 10 ng amounts of 8OH2'dG injected on the system.

To determine whether 2'dG could be oxidized to 8OH2'dG and artifactually elevate values, timing protocols were designed to allow 500 ng of 2'dG to reside on the first C18 column for 2–20 times the normal analytic time before measuring 8OH2'dG.

Preparation of urine samples

Urine samples were thoroughly vortexed at room temperature and diluted 1:1 with 8% MeOH, 0.1 M lithium acetate, pH 6.4. The diluent buffer matches the composition of MP A and dissolves particulates that coprecipitate 8OH2'dG (see Results). The minimal urine volume required was 30 μ l. Aliquots of 50 μ l of the mixture were analyzed. Urine pools of approximately 2.5 ng/ml (low, QC1 pool) and 8.5 ng/ml (high, QC2 pool) 8OH2'dG were created from preliminary studies. The pools contained control, AD, ALS, PD, and CP urine samples to reflect anticipated sample complexity. Aliquots of 1 ml of the pools were stored at -70°C and used as required for quality control for all urine assays. Pools were also created for stability studies. Separate pools "Fenton pools" were made to 1 μM Fe^{3+} , 10 μM ascorbate, and 1 μM EDTA for evaluation of possible positive artifacts.

Preparation of urine dried on filter paper

Strips of filter paper (approximately 0.6×5 cm) on which urine had been dried were placed in a Millipore Ultrafree 0.22- μm centrifugal filter tube (Millipore Corp., Bedford, MA, USA). The strips were saturated three times with 220 μ l of MP A and centrifuged after each saturation ($12,000 \times g$ for 5 min). Aliquots of 80 μ l of the filtrate were analyzed for 8OH2'dG; 200 μ l was used for creatinine determination. Aliquots of 100 μ l of each preparation were combined for a quality control pool. For control materials, S&S#903 filter paper sampling strips from the New England Regional Newborn Screening Program were saturated with a pool of urine samples from CP and control children, and dried. For assay control, some were stored at -70°C . For stability studies, others were maintained at room temperature. Thirty filter paper strips were also saturated and dried with previously assayed urine samples from children with 8OH2'dG/creatinine values ranging from 2.8 to 12.6 ng/mg.

Preparation of other matrices by precipitation and concentration

Aliquots of 500 μ l of plasma were precipitated with 1 mM LiOH in MeOH (500 μ l) and centrifuged for 10 min

at $12,000 \times g$. The pellets were resuspended in 500 μ l of MeOH and centrifuged for 10 min at $12,000 \times g$, and the supernatants were combined and evaporated under vacuum centrifugation. The residues were reconstituted in 100 μ l of mobile phase A adjusted to pH 7.2 with LiOH. Aliquots of 80 μ l were analyzed. Culture matrix was treated identically to plasma. Food, feces, and tissue were homogenized in a Waring blender with an equal volume/weight of distilled deionized water. Aliquots of 1–3 ml of the slurry were then processed as plasma. Pools of plasma, food, feces, and culture matrix and spiked pools at approximately 5–10 \times basal levels were prepared aliquoted and stored at -70°C for use as quality control materials. Separate pools of all materials and Fenton pools were prepared for stability studies.

Preparation of other matrices by SPE concentration

Diazem C18 500 mg SPE columns (Diazem Corp., Midland, MI, USA) were used under gravity flow. The columns were preconditioned sequentially with 3 ml 100% MeOH, 1 ml water, and 1 ml of phosphate-buffered saline (PBS), pH 7.2. Aliquots of 0.5–1 ml of plasma were loaded on the column, washed with 3 ml of PBS and 1 ml of water, and eluted with 1 ml of 27% Acn. The eluent was evaporated under vacuum centrifugation and reconstituted in 100 μ l of mobile phase A. Aliquots of 80 μ l were used for analysis. Other sample matrices were treated identically except for sample volume (CSF 2–3 ml, saliva 3–6 ml, sweat 3–6 ml, kidney dialysis fluid 5 ml, packed red blood cells lysed 1:1 with distilled deionized water 2 ml, tissue homogenates 1–3 ml, and microdialysates 4–5 ml). Pools and spiked pools of all materials were created as above for quality control and stability studies.

Preparation of calf thymus DNA

Calf thymus DNA digests were prepared as follows: 50–70 μg of DNA dissolved in 200 μ l of water was incubated with 7 μ l of nuclease P1 (1100 U/ml in 25 mM sodium acetate, containing 1 mM zinc chloride, pH 4.8) at 37°C for 60 min. Five microliters of alkaline phosphatase (750 U/ml in 100 mM Tris-HCl, pH 8.0) were added and the mixture was incubated at 37°C for 30 min. Aliquots of 80 μ l were used for analysis. Aliquots of 200 μ l of 2'dG and 2'dG5MP at 20 $\mu\text{g}/\text{ml}$ were carried through the preparative procedure as blanks for artifactual production of 8OH2'dG.

Creatinine assay

The Stanibo creatinine procedure number 400 (Stanibo Laboratory Inc., San Antonio, TX, USA) was

used for urinary creatinine assays. For filter paper extracts, the volume of the sample was adjusted to 150 μ l from the 30 μ l required for urine to compensate for the dilution in the extraction process.

Quality control protocols and measures

The quality control (QC) measures and calculation protocols were chosen to reflect the major objectives of validating the precision and accuracy of the methodology for long-term longitudinal studies of 8OH2'dG. All samples were assayed in an analytic sequence beginning and ending with a 10-ng/ml control standard to determine the slope of the response. All values for samples and QC test samples within a sequence were calculated independently from the bracketing average of the calibration standard. Each sequence contained a pool, a duplicate, a spiked sample, and a second standard at various levels to check the assumption of linearity of regression (regression standard). For matrices requiring preparative concentration, each sequence contained, in addition, a spiked pool and standard recovery sample (SPE recovery standard) to correct initial values for recovery. The length of the analytic sequence was determined primarily by the effect of matrix on the retention time drift and settings of gate 1 on the C8 column. For urine, the sequence was control standard, eight samples, alternating QC1 and QC2 pool, five samples, duplicate of sample 1, 10 ng/ml spike of sample 2, regression standard alternating 0.5, 1.5, 5, and 15 ng/ml, control standard. Sequences for preparations of other matrices, particularly plasma or food with high residual lipid levels, were shorter, with intervals of nine assays between control standards. Analytic runs were typically 4–10 sequences or 37–99 analyses. Sequences for DNA digests were identical to urine except that control standards, regression standards, and spiked samples for both 2'dG and 8OH2'dG were included in each sequence.

RESULTS

Limits of detection, linearity, precision, and recovery

To estimate the limit of detection (LOD), data from six studies were combined. Relative standard deviation (rsd) in percent was used as the precision estimate to describe the data. The best estimate of LOD was as a function of precision at any concentration level following $\text{rsd} = \pm(0.91 + 0.34/C)\%$, where C is the concentration of the standard in ng/ml. The LOD, determined by the constant of the second term, which varied in the six evaluations between 0.14 and 0.52, was from 7 to 21 pg/ml (350–1050 fg on column) at $\pm 21\%$ rsd. The correlation coefficient of response vs. concentration from

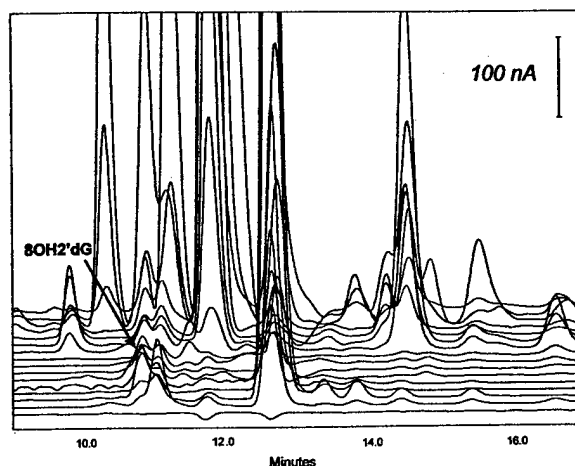


Fig. 3. Interfering substances for 8OH2'dG in a urine pool from control subjects. The gradient array chromatogram [39] represents the separation of the urinary compounds from the pool in the 1.2-min cut from the C8 column containing 8OH2'dG. In this particular sample, 36 peaks were resolved with heights greater than 10 nA in the range of cell potentials characteristic for 8OH2'dG (dominant channel 8). The complexity of the patterns and the variation among individuals and disorders illustrates the requirements for 8OH2'dG isolation.

the LOD to 100 ng/ml varied from 0.992 to 0.999. In six evaluations of repetitive injections of 10-ng/ml standards over 24 h, precision varied from $\pm 0.51\%$ to $\pm 1.14\%$ rsd, and the precision of T4/T3 ratio measurements from $\pm 3.12\%$ to $\pm 7.92\%$ rsd.

System recovery for 1 and 10 ng of 8OH2'dG (304.7 and 3407 nQ, respectively, for a one-electron transfer) was 324.2 ± 14.1 nQ and 3386 ± 44 nQ from the first C8 column. From the second C18 column, through the entire switching procedure, recovery was 301.1 ± 15.9 nQ and 3433 ± 53 nQ (mean \pm SD, $n = 3$). Studies of 2'dG conversion to 8OH2'dG on the system showed no increase from a basal level of 11 pg of 8OH2'dG/500 ng of 2'dG for times up to 3 h on the first column of the system.

The nature of the analytic problem

The critical issue for 8OH2'dG assay in urine and other tissues is avoiding artifacts related to interferences. The analytic system described here addressed this issue primarily by using the unique retention characteristics of the carbon columns to wash out any interferences co-eluting from the C8 column. Secondly, the gate on the C8 column was used to reduce the number of potential interferences, and the resolution characteristics of the C18 column were used to separate any that passed the first two steps. An example of the process is shown in Figs. 3 and 4. In this example, a urine sample from a control pool was injected onto the C8 column, and the

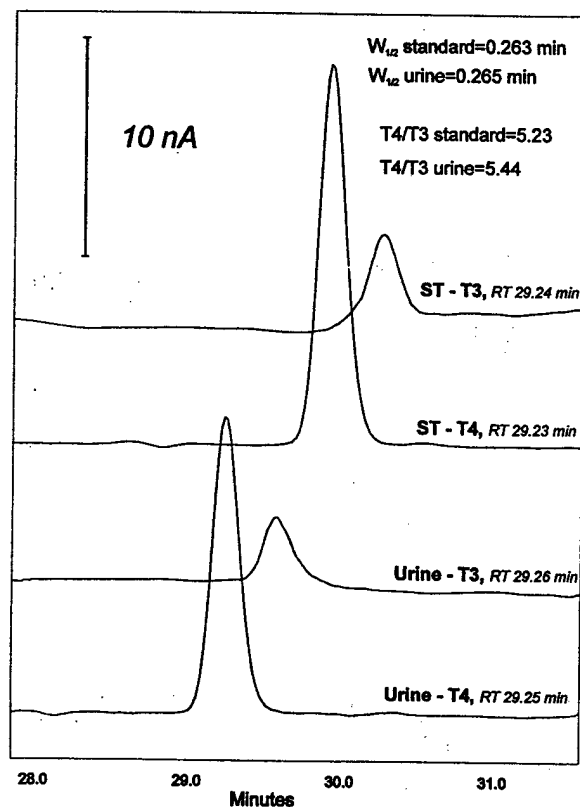


Fig. 4. Chromatograms of a 10 ng/ml 8OH2'dG control standard (ST) and a control urine pool on T3 and T4 sensors and qualitative assurance measures. This figure illustrates the effectiveness of the elution of potential interferences shown in Fig. 3 from the carbon columns. Of the 36 compounds, only 8OH2'dG was retained on the column. The three measures of qualitative peak purity for 8OH2'dG are also illustrated. T4/T3 = peak height response ratio; $W_{1/2}$ = peak width at half height; RT = retention time (separately for each sensor). The traces are shown offset for clarity.

effluent was collected from gate 1 at 11.2 to 12.4 min (the band containing 8OH2'dG with a base peak width of 0.6 min). The effluent was concentrated by vacuum centrifugation to give a volume equivalent to the original sample. The resulting concentrate was analyzed on a 16-channel coulometric array system in a 30-min linear gradient from 1% to 4% Acn in 0.1 M lithium acetate, pH 3.3, with the detector set from 0 mV to 480 mV in 32-mV increments. As shown in Fig. 3, using this approach, 36 peaks were resolved in the gradient with peak heights greater than 10 nA. This represents 36 potential interferences of 1–100 times the expected 8OH2'dG concentration in the band where 8OH2'dG elutes. The number of peaks resolved using this approach for control urine samples ranged from 18 to 41, and for urine samples from PD, AD, and ALS pools, up to 68.

The effect of the carbon trapping and washing protocol on reducing sample complexity with standard timing conditions and gates is shown in Fig. 4. This compares

the retention time, $W_{1/2}$, and the ratio response of the T3 and T4 sensors on the C18 column for a standard and the same control urine pool shown in Fig. 3. In this example, potential interferences have been almost completely eluted from both the series carbon columns or retained on the first carbon column.

Qualitative assurance measures of the 8OH2'dG peak

As shown in Fig. 4, the three qualitative assurance measures for the 8OH2'dG peak in urine samples vs. standards were in the agreement of the response ratio of T4/T3, $W_{1/2}$, and retention time. There were limitations of the qualitative assurance measures. The T3 sensor acquired only 20% of the signal and had inherently lower signal-to-noise ratio. This limited the concentration range over which a precise ratio could be obtained to values above approximately 0.5 ng/ml. In practice, the values for 8OH2'dG were calculated independently for T3 and T4. The value from T4 was taken as the analytic value. For a peak to be categorically accepted as 8OH2'dG, the value calculated from T3 had to agree within $\pm 15\%$ above 3 ng/ml and $\pm 25\%$ between 0.5 and 3 ng/ml, and have no detectable signal on T3 below 0.5 ng/ml. Peak width at half height had to agree with the standard within $\pm 8\%$, and retention time had to agree with the standard within $\pm 0.5\%$.

Studies of variation of analytic system conditions

A number of combinations of mobile phases and carbon columns were found to provide results for standards and urine pools analytically equivalent to those obtained with the standard conditions used. 8-Hydroxy-2'-deoxyguanosine peaks that passed the qualitative assurance tests, comparing standards and pools of different samples, were obtained with MeOH levels in MP A below 6% and pH values between 4–7, and Acn levels in MP B below 5% and pH values below 4. Concentration levels for the pools were identical within the precision of the system when the qualitative assurance measures were met. The finding of equivalent analytic values in multiple conditions provided an initial level of qualitative assurance for 8OH2'dG in the pools of samples from patients with different disorders.

The final analytic conditions represent a compromise that balances qualitative certainty, precision, and sensitivity, against assay speed and long-term stability. The major compromises considered when finalizing the assay conditions were (i) lower organic modifier in MP A reduced the number of possible interferences transferred to the carbon columns but increased assay time and decreased the first column stability to backflush; (ii)

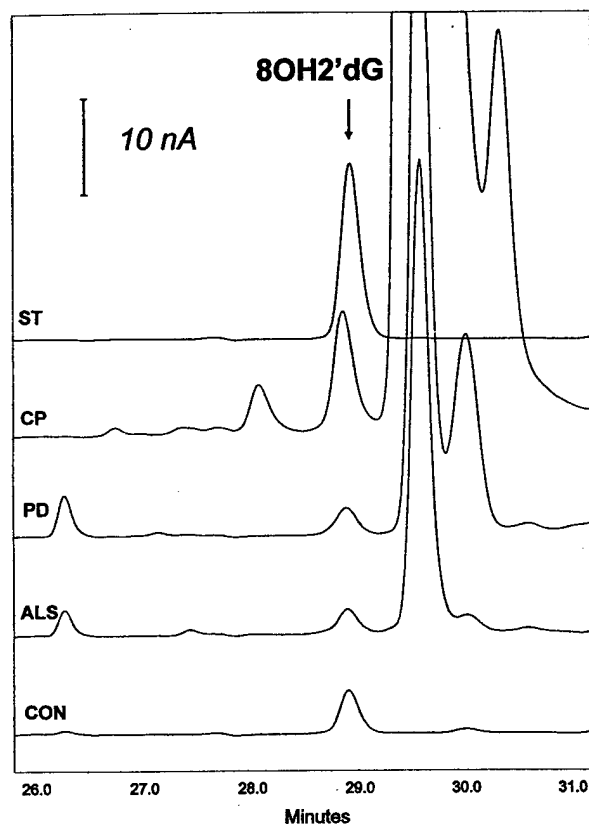


Fig. 5. Chromatograms of urine samples from a control subject (CON), and from patients with ALS, PD, and CP. ST = a 10 ng/ml 8OH2'dG control standard. Only T4 is shown for clarity. These particular chromatograms of patients with ALS, PD, and CP have been among the most complex ones encountered in this study. They also illustrate the effectiveness of carbon columns in achieving selective separation for 8OH2'dG from disorder-related interferences. Compounds eluting after the carbon-washing step can be inferred to have structural similarity to 8OH2'dG.

shorter gate 1 intervals decreased possible interferences trapped, but also decreased stability of response over long periods; (iii) longer carbon columns allowed more interferences to be removed, but increased assay time and slightly decreased sensitivity; and (iv) dissimilar organic modifiers and pH of MP B and MP D, or lower concentrations of adenosine in MP D allowed more control of the carbon elution step, but decreased sensitivity.

Use of timing and potential variables with final assay conditions

In this study, we encountered 11 samples that failed the qualitative measures of peak response for 8OH2'dG (three urine samples from patients with CP, one urine sample from a patient with AD, five rat urine samples obtained using metabolic cages, one sample of a food pool, and one plasma sample from a patient undergoing

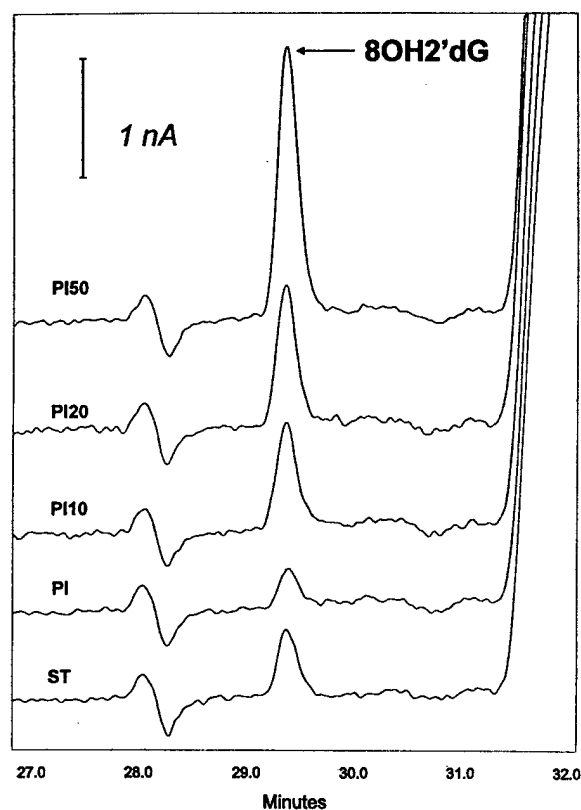


Fig. 6. 8-Hydroxy-2'-deoxyguanosine in plasma after SPE procedure. ST = 10 pg/ml SPE recovery standard; PI = plasma pool from control subjects at 4.8 pg/ml; PI10, PI20, and PI50 = 10, 20, and 50 pg/ml spikes of the pool, respectively (only T4 is shown). These chromatograms illustrate the typical >95% recovery of SPE procedure developed. The lower level of complexity in plasma as compared with urine is illustrated by the similarity of the sample and the standard chromatogram patterns. Original volume of the samples was 0.8 ml, and the concentration factor was 8.

kidney dialysis). These were subsequently successfully assayed under different timing conditions. By reducing the time interval of gate 1 from the standard setting of 1.2 to 0.7 min, fewer potential interferences were transferred to the carbon columns. By increasing the flush volume of the carbon columns to 18 ml (as opposed to regularly used 9–12 ml), more potential interferences were removed. By narrowing the range of the cell 2 sensor settings (e.g., setting the 5021 cell to 75 mV, T3 to 70 mV, and T4 to 120 mV), fewer potential interferences were detected. This protocol sacrificed typically 50% of sensitivity. Because of thermal- and matrix-related drift, these conditions were inherently less stable, but could be maintained with equivalent precision for 4–6 h.

Generally, for most sample types, the elution of interferences from the carbon columns was highly selective, and it was unusual to observe more than two other resolved peaks in the final chromatograms. Urine samples from patients with disorders and rat urine from

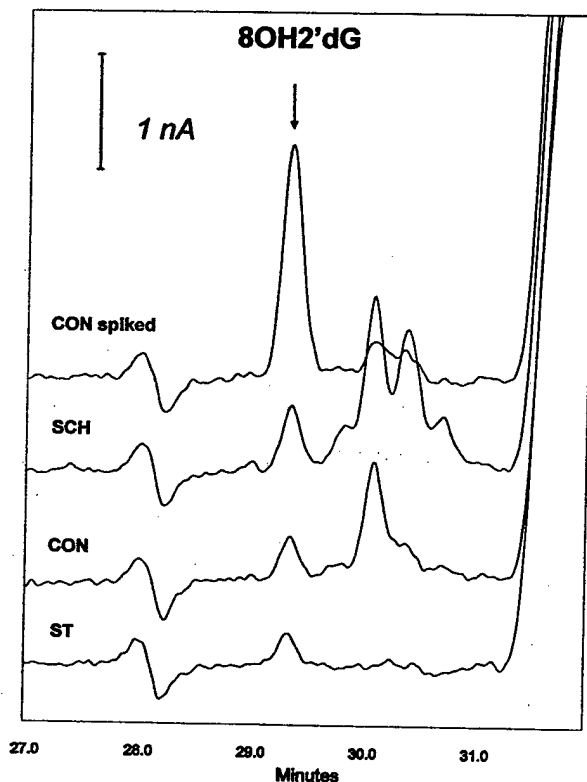


Fig. 7. 8-Hydroxy-2'-deoxyguanosine in CSF after SPE procedure. ST = 1 pg/ml SPE recovery standard; CON = CSF pool from control subjects at 1.18 pg/ml; SCH = CSF sample from a schizophrenia patient at 1.46 pg/ml; CON spiked = 10 pg/ml spike of the control pool. Original sample volume was 2.3 ml, and the concentration factor was 23. Several additional noninterfering peaks were detected in the SCH patient.

metabolic cages contaminated with food and feces were the most complex encountered. Although 8OH2'dG peaks were completely resolved for these samples and met the qualitative certainty tests, these sample types contained up to six other resolved peaks. These observations may explain the incidence of samples of rat urine samples obtained using metabolic cages that failed the qualitative assurance tests for peak purity of 8OH2'dG.

Representative chromatograms

Typical chromatograms for various sample types are shown in Figs. 5–10. The examples in Fig. 5 were chosen to represent the most complex urine samples encountered in this study. From Fig. 5, it is apparent that disorders introduce additional complexity in the final chromatogram. The 3–5 additional peaks in various disorders resolved on the C18 column, following the initial separation on the C8 column and the carbon column-washing step, varied in magnitude on a sample-specific basis. Individual samples in a disorder category were often not

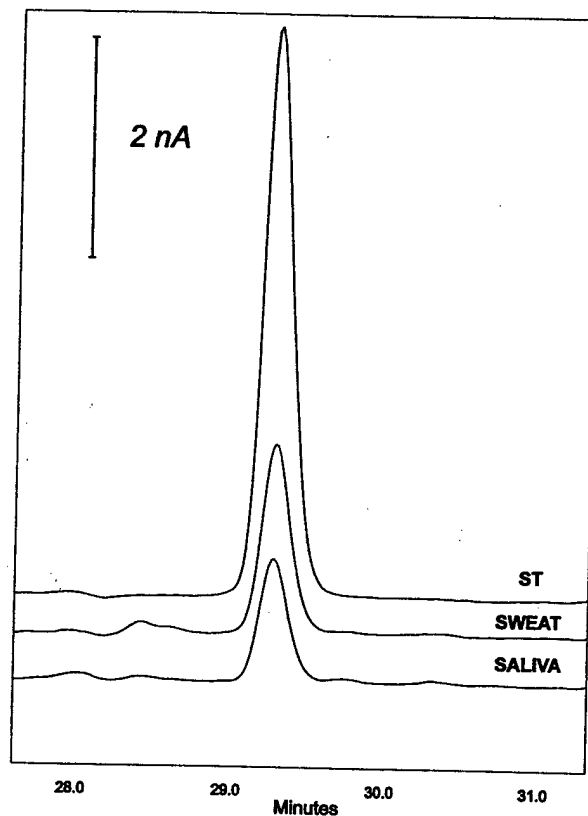


Fig. 8. 8-Hydroxy-2'-deoxyguanosine in sweat and saliva pools after SPE procedure. ST = SPE recovery standard at 100 pg/ml. Original samples volume was 6 ml, with a concentration factor of 60. This illustrates the similarity of sweat and saliva to plasma in terms of the complexity relative to urine.

significantly more complex than control samples, which also varied in complexity. The small peak at 30 min in control samples varied in magnitude up to 10 times that shown in this example.

Plasma, sweat, saliva, and microdialysates were virtually indistinguishable from control standards in most samples, as shown in Figs. 6, 8, and 9. This was unexpected, because the region where 8OH2'dG elutes on the C8 column was of comparable complexity for these types of samples and for urine.

Cerebrospinal fluid, as shown in Fig. 7, had additional resolved peaks related to specific disorders. There was also a subtle effect of the SPE procedure for CSF on the level of a peak at 30 min in control pools, as demonstrated in this example for the pool and spiked pool.

The chromatogram of a DNA digest, shown in Fig. 10, illustrates the effectiveness of the method in reducing baseline artifacts and drift in the measurement of the 8OH2'dG that has been observed in our laboratory on a sample-specific basis in direct isocratic assays (note the detector was modified for 1-mA full scale response for T1 and T2).

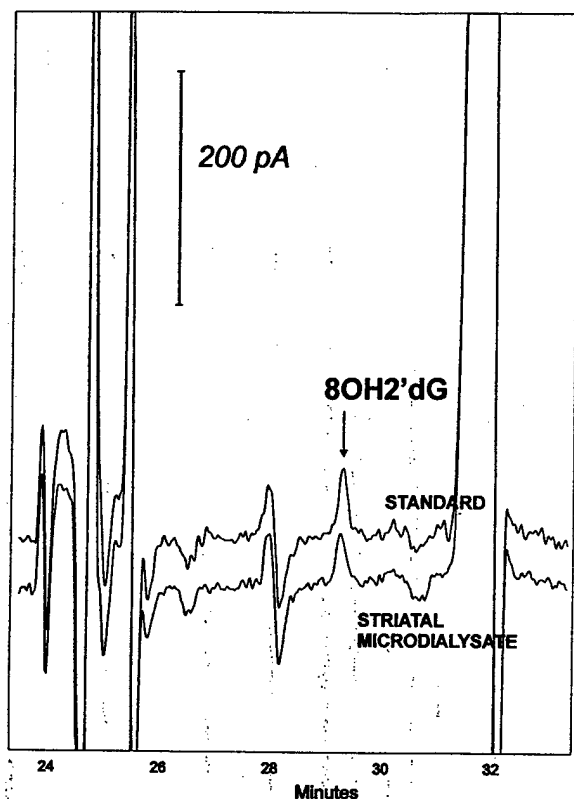


Fig. 9. In vivo production of 8OH2'dG in the rat brain, as measured using brain microdialysis. A concentric microdialysis probe (200 μ m outer diameter, length 4 mm, molecular weight cutoff 18 kDa) was implanted into the striatum 24 h before the perfusion experiments. The probe was perfused with Ringer's solution at 10 μ l/min. Dialysate was collected for 8 h, and the entire resulting volume was concentrated, using essentially the same SPE protocol as described for plasma samples. Chromatograms shown are: rat striatal microdialysate at approximately 0.38 pg/ml and SPE recovery standard at 0.4 pg/ml. The concentration of the SPE recovery standard was chosen to match the level of 8OH2'dG anticipated. Although chromatogram has no other peaks, the application approaches the limitations of sensitivity of the system and methodology.

Levels of 8OH2'dG and quality control evaluations

Table 1 presents values of 8OH2'dG in the matrices studied. The values cited for humans are only for control individuals who were not run as control subjects for studies of disorders. The values cited for animals are for controls. The values for (*n*) reflect single individuals or single samples and not multiple samples on the same individual or multiple assays of the same base sample.

Tables 2–4, summarize the quality control data for assays performed under the fixed conditions described. The data are abstracted from 473 assay sequences (approximately 5200 analyses) performed over a 1-year period for the matrices discussed. In this summary, 13 assay sequences (188 analyses) were rejected from evaluation because of hardware failure (typically pump or valve component failure, loss of temperature

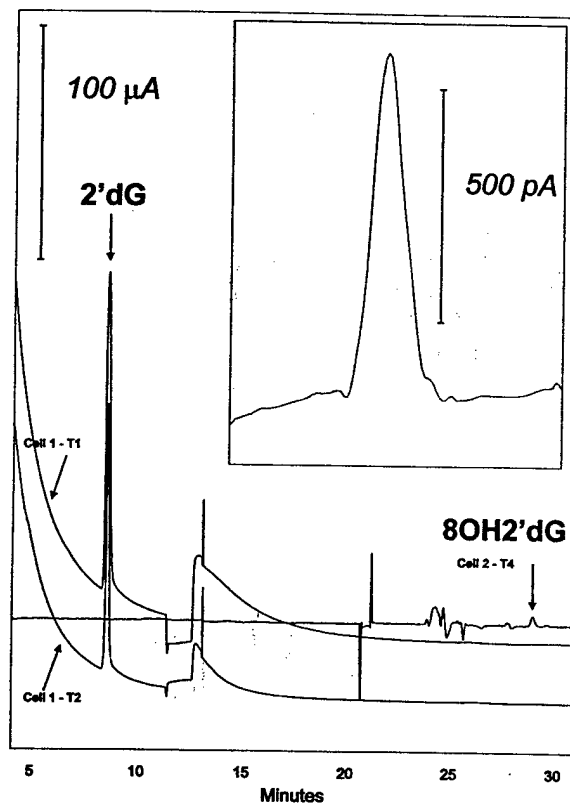


Fig. 10. 2'-Deoxyguanosine and 8OH2'dG in calf thymus DNA. The response characteristics for 2'dG on T1 and T2 sensors and the effect of reducing baseline artifacts on T4 for 8OH2'dG are shown. Inset: 8OH2'dG at T4.

control, and overpressure or loss of resolution on the C8 column). No values were rejected for other reasons. The QC data for urine assays covers the entire 1-year period, and 7 months for plasma and other matrices. The QC data include only assays performed under the fixed routine protocols and conditions described, and not assays made in the initial validation of procedures. During this study, column pressures on the C8, C18, and the carbon columns were kept within $\pm 15\%$ of their initial value. The retention time of 8OH2'dG on the C8 column and time settings of gate 1 were evaluated biweekly, and the retention times of the carbon columns and flushing rate settings were evaluated monthly. Over the entire period of this study, carbon columns were changed once, the C8 column was changed six times, and the C18 column was changed twice. No component changes were required during routine assays of urine, but component changes were required after assays of plasma, foods, CSF, culture matrix, or tissue preparations. Data analysis parameters gave unacceptable baselines on 3% of samples requiring adjustment of parameters or manual assessment.

Table 1. 8-Hydroxy-2'-Deoxyguanosine Levels in Various Biologic Matrices

	8OH2'dG, Mean \pm SEM (n)	8OH2'dG, Range	8OH2'dG/Creatinine, ng/mg, Mean \pm SEM (n)	Urinary 8OH2'dG output, ng/kg/24 h, Mean \pm SEM (n)
Urine, ng/ml				
Adult controls, males	4.09 \pm 0.31 (104)	0.32–13.4	3.68 \pm 0.047 (104)	75.66 \pm 0.44 (36)
Adult controls, females	3.39 \pm 0.35 (96)	0.41–11.3	3.96 \pm 0.038 (96)	72.06 \pm 0.76 (35)
Age 3–9 years, controls	4.09 \pm 0.22 (32)	0.64–12.9	4.62 \pm 0.091 (32)	
Neonates (50–63 days, filter paper)		2.74–33.4	13.39 \pm 0.082 (150)	
Rhesus monkeys	5.64 \pm 1.08 (12)	2.13–9.98	6.23 \pm 0.14 (12)	
Rats	6.19 \pm 0.38 (28)	1.48–6.34	7.81 \pm 0.093 (28)	123.60 \pm 11.41 (12)
Mice	7.34 \pm 0.64 (22)	1.34–10.45	6.90 \pm 0.759 (22)	
Other matrices, pg/ml				
Plasma, adult controls	13.40 \pm 2.11 (28)	4.28–21.19		
Red blood cells, adults	2.1 \pm 0.31 (6)	1.28–2.45		
CSF, adult controls	0.98 \pm 0.03 (16)	0.64–1.54		
Saliva, adult controls	15.30 \pm 3.36 (6)	6.72–18.36		
Sweat, adult controls	11.22 \pm 9.50 (8)	0.32–47.27		
Kidney dialysate	67.34 \pm 20.31 (10)	5.81–164.2		
Rat brain microdialysate	0.32 \pm 0.03 (4)	0.22–0.36		
Rat muscle microdialysate	0.19 \pm 0.04 (4)	0.16–0.21		
<i>C. elegans</i> cell culture	11.24 \pm 5.36 (41)	3.11–21.42		
Food (pg/g)	34.09 \pm 7.51 (16)	6.32–79.38		
Rat feces (pg/g)	0.78 \pm 0.04 (12)	0.34–1.15		
Calf thymus DNA (8OH2'dG/10 ⁵ 2'dG)	5.92 \pm 0.62 (11)	4.58–8.28		

External and internal blind testing

A set of eight blind duplicate urine samples from NIOSH subaliquoted at the time of acquisition gave precision of $\pm 4.11\%$ rsd at a mean level of 4.13 ng/ml. Fifty internal blind duplicates subaliquoted at the time of initial assay and reanalyzed at random intervals of 1–12 months gave precision of $\pm 4.91\%$ rsd at a mean level of 3.98 ng/ml. Five spiked samples made in a single urine pool at a base level of 3.70 ng/ml, and 54 duplicate samples from subaliquots of 24-h urine samples stored at -70°C were submitted in a batch of 570 other samples provided by RJ Reynolds Tobacco Inc. over a 1-year period. The target values of the spiked samples vs.

(found values) were 4.18 (4.19), 8.51 (8.12), 13.27 (12.74), 22.65 (22.43), and 40.87 (40.94) ng/ml. The correlation of target vs. found values was $r = .992$, with an SE of estimating target from found values of ± 0.121 ng/ml. The average recovery was 98.2%, and found values were within $\pm 4.6\%$ of target values at all five levels. The data indicated an assay rsd of less than $\pm 2\%$. The precision of duplicates from the 54 samples was $\pm 9.01\%$ rsd at the level of 3.06 ng/ml. Two of the paired samples 6.04 vs. 3.22 and 3.13 vs. 1.32 ng/ml contributed the majority of the rsd. Eliminating the two samples gave precision of $\pm 6.78\%$ rsd at 3.04 ng/ml. The outlier samples were reanalyzed, and the values were confirmed

Table 2. Precision and Quality Control Data for Urinary 8OH2'dG Methods

	Mean \pm % rsd (n)	
	Urine, ng/ml	Filter paper urine, ng/mg creatinine
10 ng/ml control standard	9.99 \pm 2.63 (341)	10.08 \pm 2.13 (14)
0.1 ng/ml regression standard		0.103 \pm 4.67 (5)
0.3 ng/ml regression standard		0.295 \pm 3.83 (5)
0.5 ng/ml regression standard	0.506 \pm 3.41 (84)	0.502 \pm 3.21 (5)
1.5 ng/ml regression standard	1.491 \pm 3.14 (84)	
5 ng/ml regression standard	5.058 \pm 2.78 (85)	
15 ng/ml regression standard	14.92 \pm 2.65 (85)	
QC1 urine pool	2.62 \pm 6.13 (171)	3.64 \pm 7.15 (16)
QC2 urine pool	8.21 \pm 4.12 (170)	
Urine duplicates*	4.05 \pm 4.19 (341)	11.94 \pm 7.13 (17)
Recovery of 10 ng/ml standard spike in urine	10.14 \pm 5.23 (331)	

* The mean value for all duplicates of urine samples analyzed.

Table 3. Precision and Quality Control Data for 8OH2'dG Methods Requiring Preparative Protocols

	Mean \pm %rsd (n)	
	SPE Protocol	Precipitation Protocol
10 ng/ml Control standard	10.05 \pm 2.36 (61) ^a	9.76 \pm 5.93 (36) ^a
0.1 ng/ml Regression standard	0.103 \pm 4.56 (20) ^a	0.97 \pm 5.34 (14) ^a
0.3 ng/ml Regression standard	0.289 \pm 4.88 (16) ^a	0.311 \pm 5.62 (11) ^a
0.5 ng/ml Regression standard	0.493 \pm 4.31 (16) ^a	0.501 \pm 4.95 (11) ^a
SPE recovery standard 0.1 pg/ml	0.094 \pm 20.51 (8) ^b	
SPE recovery standard 1 pg/ml	0.95 \pm 7.22 (11) ^b	
SPE recovery standard 10 pg/ml	9.62 \pm 5.24 (12) ^b	
SPE recovery standard 50 pg/ml	48.18 \pm 5.12 (19) ^b	
SPE recovery standard 100 pg/ml	97.23 \pm 4.13 (11) ^b	
Plasma pool	13.82 \pm 6.98 (14) ^b	14.62 \pm 14.69 (16) ^b
Plasma duplicates	15.71 \pm 7.21 (14) ^b	9.81 \pm 15.76 (21) ^b
Recovery of 50 pg/ml spike in plasma	47.11 \pm 8.14 (14) ^b	51.34 \pm 13.62 (16) ^b
CSF pool	0.96 \pm 8.32 (21) ^b	
Recovery of 10 pg/ml spike in CSF pool	10.19 \pm 6.77 (11) ^b	
Sweat pool	53.45 \pm 4.54 (4) ^b	
Recovery of 50 pg/ml spike in sweat pool	47.43 \pm 6.23 (6) ^b	
Saliva pool	14.52 \pm 6.75 (7) ^b	
Recovery of 50 pg/ml spike in saliva pool	48.38 \pm 6.23 (7) ^b	
Kidney dialysate pool	78.59 \pm 5.83 (8) ^b	
Kidney dialysate duplicate	102.39 \pm 4.98 (8) ^b	
Recovery of 100 pg/ml spike in dialysate pool	97.24 \pm 7.23 (8) ^b	
Food pool		41.22 \pm 13.18 (13) ^c
Food duplicates		67.18 \pm 9.98 (13) ^c
Recovery of 100 pg/g spike in food pool		97.44 \pm 11.69 (12) ^c
<i>C. elegans</i> culture pool		13.33 \pm 9.92 (12) ^b
Recovery of 50 pg/ml spike in <i>C. elegans</i> culture pool		52.41 \pm 11.76 (12) ^b

8-Hydroxy-2'-deoxyguanosine levels are presented as: ^a ng/ml, ^b pg/ml, ^c pg/g.
For duplicates, the mean values for all duplicates of samples are presented.

to be within $\pm 4\%$ of the original value. The sample with high 8OH2'dG values of each pair was found to have approximately 90% more precipitate, which contained from 32% to 48% of the 8OH2'dG.

Preparative and sample handling factors affecting the 8OH2'dG assays

One of the main problems encountered in urine assays was the sample-specific inclusion of 8OH2'dG in precipitates that form on storage or repetitive freeze-thawing of samples. This was examined and controlled in a number of studies throughout the evaluation. A representative group of samples showing the variability of this effect is shown in Table 5. Creatinine, unlike 8OH2'dG, showed no difference in values between supernatants and thoroughly vortexed samples. Dilution in neutral buffer will recover the 8OH2'dG from precipitates. However, significant errors can occur from exclusion of 8OH2'dG in precipitates, if stored samples are not thoroughly homogenized before subaliquoting.

The most important factor affecting the assay of urine dried on filter paper was the differential elution of creatinine and 8OH2'dG from the filter paper matrix. All of the creatinine, but only 80–85% of the 8OH2'dG eluted

in the first 220 μ l of MP A extract. Three consecutive extractions were required for equivalent recovery of both analytes, which put the concentration of 8OH2'dG into a less precise range. The correlation of 30 bulk and filter paper urines prepared in-house, over the range of 2.8–12.2 ng 8OH2'dG/mg creatinine, was $r = 0.991$. The standard error of estimating filter paper values from bulk

Table 4. Precision and Quality Control Data for 8OH2'dG Method in DNA Digests

	Mean \pm %rsd (n)
10 ng/ml control 8OH2'dG standard	10.11 \pm 1.98 (48) ^a
0.1 ng/ml regression 8OH2'dG standard	0.101 \pm 5.64 (13) ^a
0.3 ng/ml regression 8OH2'dG standard	0.293 \pm 5.72 (13) ^a
0.5 ng/ml regression 8OH2'dG standard	0.501 \pm 4.15 (13) ^a
50 μ g/ml control 2'dG standard	50.13 \pm 3.69 (45)
10 μ g/ml regression 2'dG standard	10.72 \pm 3.21 (12)
100 μ g/ml regression 2'dG standard	98.93 \pm 4.13 (12)
150 μ g/ml regression 2'dG standard	147.32 \pm 4.42 (12)
200 μ g/ml regression 2'dG standard	197.32 \pm 4.12 (11)
DNA digests pool	8.31 \pm 7.23 (22) ^b
DNA digests duplicates	11.32 \pm 6.88 (21) ^b
Recovery of 200 pg/ml 8OH2'dG spike in DNA digests	204.52 \pm 6.44 (15) ^b

8-Hydroxy-2'-deoxyguanosine levels are presented as: ^a ng/ml, ^b 8OH2'dG/10⁵ 2'dG.
2'dG levels are presented as μ g/ml.

Table 5. Effect of Urinary Particulate Materials on 8OH2'dG Measurements in Urine (ng/ml)

Sample	1:1 Dilution with buffer		Centrifugation at +4°C		Centrifugation at +25°C	
	Supernatant	Precipitate	Supernatant	Precipitate	Supernatant	Precipitate
Urine 1	8.341	ND	7.283	0.914	7.939	0.466
Urine 2	5.280	ND	3.620	1.436	4.104	1.136
Urine 3	4.042	ND	4.143	0.035	4.011	ND
Urine 4	4.003	ND	3.104	1.034	3.704	0.331
Urine 5	3.104	ND	2.486	0.581	2.843	0.311
Urine 6	1.063	ND	0.163	0.980	0.524	0.617
QC1, low pool	2.446	ND	2.115	0.285	2.401	0.113
QC2, high pool	8.463	ND	5.692	2.351	7.259	0.978
UA, 8OH2'dG ^a	4.938	ND	2.146	2.984	4.103	0.801

8-Hydroxy-2'-deoxyguanosine levels are presented in ng/ml.

^a Solution of uric acid, saturated at +40°C, containing 5 ng/ml 8OH2'dG.

ND = not detected.

values was ± 0.672 ng/mg. The main contribution to the intermethod error was from the lower combined precision of the 8OH2'dG and creatinine in the more dilute filter paper preparations.

The filter paper urine method was evaluated on a set of 150 Japanese neonatal filter paper urine samples, as shown in Table 2. The absorption of 8OH2'dG to the filter paper matrix raised the possibility of differential distribution of creatinine and 8OH2'dG across the filter matrix during drying. To evaluate this, five strips, representing the total available material from each of two samples of neonatal filter paper urine, with initial low and high values were analyzed. The low value sample gave a range of 8OH2'dG from 0.251 to 0.761 ng/ml and of creatinine from 0.101 to 0.305 mg/ml. The mean 8OH2'dG/creatinine ratio was 2.56 ng/mg, $\text{rsd} \pm 6.14\%$. The high-value sample gave a range of from 1.032 to 3.310 ng/ml in 8OH2'dG and from 0.034 to 0.098 mg/ml in creatinine. The mean 8OH2'dG/creatinine ratio was 34.16 ng/mg, $\text{rsd} \pm 7.14\%$. This study and the duplicate sample data indicated that differential distribution on a filter paper strip was not a problem, and that the assay was applicable to samples of dried urine on filter paper using any section of the paper.

Precipitation and SPE concentration protocols were analytically equivalent. Comparison of 26 samples of plasma from control subjects and from patients with different disorders analyzed using both approaches gave a correlation $r = .943$ over the range of 4–28 pg/ml.

The principal advantage of the precipitation/evaporation protocol was its simplicity. For small sets of samples of control plasma, and for food slurries and *C. elegans* culture media, where sample concentrations are approximately 10 pg/ml, it provided adequate concentration for detection on the system. The primary limitation for larger sets of plasma samples was inclusion of variable amounts of lipids in the preparation. This affected the performance of the C8 column, and as a result, caused

variability in the settings of gate 1. This made typical automated runs of 24–48 h risky and reduced precision, as shown in Table 3. For food and culture matrix, this was not a problem; however, for the other sample types, matrix carryover effects and concentration levels of 0.2–10 pg/ml made precipitation and evaporation impractical, except for estimation of levels. Precipitation could not be used for kidney dialysis fluid because of the coprecipitation of 8OH2'dG with high levels of uric acid.

The SPE concentration protocols allowed quantitative concentration factors up to 200. The principal problem in their use was operational. Plasma, saliva, red blood cell lysates, or CSF samples could not be allowed to sit on the column for more than 10–15 min before washing with buffer. Time-dependent binding of matrix components to the SPE packing reduced recoveries by up to 30%.

The issue of internal standards

The high selectivity of the column-switching procedure with carbon columns, which is its principal advantage, conversely creates a problem in the use of internal standards. Although unidentified compounds from some samples clearly pass through the C8 column separation and carbon column washing step and are resolved on the C18 column, we were unable to find any authentic standard reference material that did so. The lack of an internal standard does not pose a problem in analysis of urine, because the preparative step involves only dilution. For matrices requiring SPE or precipitation procedures for concentration and protein removal, the inability to have an internal standard forces reliance on pools, spiked pools, and spiked samples to assess recovery. The resolution of the analytic system allows aggressive conditions for quantitative recovery; however, there is no absolute assurance that an individual sample and a spike of that sample, even when run at the same time; will behave identically. Indeed, there is no absolute certainty

that an authentic reference internal standard that behaved like 8OH2'dG through the chromatographic protocol would have protein or surfactant ligand characteristics to mimic the behavior of 8OH2'dG in all samples.

The use of isotope-labeled internal standards, which is outside the scope of the current effort, could provide additional certainty relative to levels of recovery at all points in the analytic process. Unlike GCMS and LCMS techniques, which can monitor mass peaks for C13-labeled internal standards, only β -emitting C14-labeled 8OH2'dG would be compatible with the liquid chromatography separation technology. This approach, like the more common overspiking of samples, still would have to address the possibility of slow equilibration of the isotope-labeled compound with endogenous 8OH2'dG bound to possible ligands. The use of isotope-labeled standards with LCEC technology would primarily be viewed as a tool for additional validation of preparative protocols and instrument behavior. The requirement of using radioactive isotope-labeled compounds as internal standards, as opposed to C13-labeled compounds, is an inherent limitation of direct LC methods. Whether they could be economically and efficiently incorporated into routine assays as with GCMS or LCMS technology is an open question.

Evaluation of loss and production of 8OH2'dG

Aliquots of low QC1 and high QC2 urine pools stored at room temperature and pools with added iron ascorbate and EDTA, Fenton pools, kept at room temperature and covered with filter paper to allow aeration, were run daily for 20 days. There was no measurable variation in 8OH2'dG levels over this time period. Correlation of values vs. time gave r values of -0.002 to $+0.0013$. The means and precision of values over 20 d for the QC1 and QC2 levels were 2.48 ng/ml, $\text{rsd} \pm 5.14\%$ and 8.34 ng/ml, $\text{rsd} \pm 3.78\%$, respectively, which were equivalent to that of the pools run during the entire study. The level of precipitate increased substantially in the high QC2 pool at room temperature and contained 26% of the 8OH2'dG at day 20. Creatinine values in the pools varied significantly after 3 days. Both analytes, however, were stable when dried. The 8OH2'dG/creatinine ratio did not differ within the assay variability over 30 d on dried filter paper samples at room temperature. The following pools and Fenton pools kept over 2 days at room temperature showed no loss or production of 8OH2'dG from their initial levels: plasma at 13.2 pg/ml; CSF at 1.1 pg/ml; *C. elegans* culture matrix at 13.5 pg/ml; kidney dialysis fluid at 58.0 pg/ml; rat fecal slurry (from animals 8 h after oral administration of 10 $\mu\text{g/kg}$ 8OH2'dG) at 4.2 pg/g.

Pools of other materials for the same time interval did

show increases in 8OH2'dG levels: plasma in which cellular material from the buffy coat was included, from 14.1 to 20.2 pg/ml; saliva, from 15.5 to 26.8 pg/ml; sweat, from 4.7 to 14.8 pg/ml; mixed meat and vegetable food slurries, from 32.4 pg/g to 68.3 pg/g. Interestingly, however, the same mixed food pool treated in a mock digestion process with 1 ml of saliva and 100 μg of trypsin/100 g and HCl to pH 1.5 for 4 h decreased to 21 pg/g.

Aliquots of 500 μl of 100 $\mu\text{g/ml}$ 2'dG standards were left on the SPE columns for 1–3 h. These showed no measurable change from the baseline level of 8OH2'dG in the original standard. Separate assays of 2'dG in plasma, saliva, kidney dialysis fluid, and CSF indicated normal levels on the order of 0.2 ng/ml. Consequently, 2'dG conversion is not likely to create an 8OH2'dG elevation artifact in these matrices.

The production of 8OH2'dG in DNA preparations is a unique situation because of the high levels of 2'dG. Various lots ($n = 7$) of authentic standards of 2'dG and 2'dG5MP had mole ratios of 8OH2'dG/2'dG of $15\text{--}23 \times 10^{-5}$ and $5\text{--}7 \times 10^{-5}$, respectively. The mole rate of production of 8OH2'dG from 2'dG/24 h varied substantially in different diluents: in deionized water, 0.35×10^{-5} ; in deionized double distilled water, 0.16×10^{-5} ; in mobile phase A, 0.083×10^{-5} ; in a pool of digested DNA extracts diluted 1:1 in mobile phase A at a starting mole ratio of 11.1×10^{-5} , 0.076×10^{-5} . The rate of production of 8OH2'dG from 2'dG evaluated at times up to 8 d increased exponentially and was perhaps autocatalyzed. Addition of Fe^{3+} , ascorbate, or EDTA, individually or in combination, dramatically increased the production rate at room temperature to as high as 220×10^{-5} . Standards of 2'dG at 20 $\mu\text{g/ml}$ carried through the DNA digestion protocol showed no measurable change in 8OH2'dG levels from their initial mole ratio levels of $19.27 \pm 0.52 \times 10^{-5}$ (mean \pm SEM, $n = 4$). Standards of 2'dG5MP at 20 $\mu\text{g/ml}$ showed a significant increase from a mole ratio of $6.73 \pm 0.51 \times 10^{-5}$ 8OH2'dG/2'dG5MP to $15.7 \pm 0.62 \times 10^{-5}$ 8OH2'dG/2'dG (mean \pm SEM, $n = 4$). However, the same 2'dG5MP standard, subjected to the alkaline phosphatase only, similarly showed an increase to $16.1 \pm 0.82 \times 10^{-5}$ 8OH2'dG/2'dG (mean \pm SEM, $n = 3$).

Considered primarily from the point of analyzing samples that have already been prepared, the rates of production defined acceptable times of 12 h on the autosampler for automatic analysis, and acceptable mobile phase conditions and diluents for accurate assay.

DISCUSSION

The evidence accumulated to date suggests that 8OH2'dG may be a useful systematic marker of oxida-

tive stress. The assays available, however, have required either extensive cleanup steps or very complex preparative methodology. We have presented a single set of conditions and various preparative methods for 8OH2'dG based on an LCEC column-switching system developed around the unique characteristics of carbon columns that are adaptable to a wide range of biologic matrices. For continuous automated high sample throughput, the methods are almost completely free of interference related to disorders or therapeutic intervention in the disorders and matrices studied. In the few cases where interference occurs, the qualitative assurance protocols allow detection of the errors and reassay with more qualitatively discriminative but less stable conditions.

With this system, the urinary levels of 8OH2'dG for adult controls in all measures of concentration range, creatinine corrected values, and rates of output for male and female subjects are comparable to those from prior studies using LCEC column-switching methodology [13, 20]. Mean values of urinary 8OH2'dG in controls found in this study are in the same range as those previously reported using LCEC with SPE sample preparation [30], GCMS [18], LCMS [26], and LCEC with monoclonal or polyclonal antibody isolation techniques [33,34]. They are significantly lower than those reported by other groups and methods using LCEC [12,31], and than those reported with ELISA techniques [24,25]. The use of carbon columns allowed highly selective separation of 8OH2'dG from interfering peaks. However, some resolved peaks were always present at some level in the final chromatographic step. These unidentified compounds, which have similar chromatographic behavior on the C8, C18, and carbon columns and similar electrochemical characteristics, can be inferred to have structural similarity to 8OH2'dG, and possibly, to cross-react with 8OH2'dG in ELISA assays. These observations could, in part, explain both the higher 8OH2'dG levels and differing reports of cross-reactivity in ELISA [24, 25]. In view of the quantitative recovery of 8OH2'dG in the system discussed here, the lower urinary 8OH2'dG values may simply reflect a more universal rejection of interferences by both the previously described column-switching protocol [13,20] and in this study by the adaptation of that protocol to add a carbon column trapping and washing step. Values for rat urine are also consistent with previous reports [22,28,36]. Mean values for control plasma of 13.4 ± 2.11 pg/ml (mean \pm SEM) were lower than values of 19.3 ± 4.20 pg/ml (mean \pm SEM) previously reported [33]. Recently reported values for ventricular CSF levels of 8OH2'dG in controls were 0.167 nmol/mg protein for a protein content of 1.36 mg/ml, equivalent to 64.3 ng/ml [37]. This value is 65,000 times higher than the 0.98 pg/ml 8OH2'dG levels in lumbar

CSF from control subjects observed in this study, and also inconsistent with our observations of levels of 0.36 pg/ml in rat striatal microdialysates. The origin of CSF probably could not account for these differences, because we have found levels of 1.3–1.6 pg/ml for Meccle's cave, fossa postera, and ventricular CSF from trigeminal neuralgia patients (this laboratory, unpublished data). This possibly suggests an unresolved intermethod bias between LCEC and GCMS in this application for 8OH2'dG similar to that observed for DNA digests [38]. Values for other matrices have not been previously reported in the literature.

Values for 8OH2'dG/2'dG ratios in DNA are consistent with other LCEC methods for similar samples [3]. They are, however, higher by two orders of magnitude than those reported for DNA extracts in other work [22]. They are comparable to other LCEC methods for calf thymus DNA [38], but an order of magnitude lower than those obtained by GCMS. The studies of authentic standards of 2'dG carried through the DNA digestion protocol would indicate that at the analytically detectable change in mole ratio levels of approximately 10^{-5} 8OH2'dG/2'dG there is no process effect. The increase in 8OH2'dG from 2'dG5MP standards most probably reflects the formation of 8OH2'dG from 8OH2'dG-5'-monophosphate rather than process-related hydroxylation. However, using standards to detect process-related anomalies below mole ratio levels of 10^{-5} will require authentic standards that are an order of magnitude lower in contaminating 8OH2'dG than currently available. It is clear that there is a wide range of variation in values for 8OH2'dG in DNA, related among other issues to the tissue studied, the acquisition, storage, and extraction of the samples, and the digestion protocol. Instrumental analysis, if controlled for artifactual production and loss, is likely to be the least of these issues. Until such issues are systematically resolved, 8OH2'dG levels in DNA reported in this work should be viewed as method- and laboratory-specific to a particular study.

The temporal stability of 8OH2'dG found for most matrices in this study is consistent with previous observations of both 8OH2'dG stability and lack of artifacts by production from 2'dG [28]. The low levels observed in foods in this study, coupled with prior observations of insignificant increases in urinary 8OH2'dG after oral administration in rats and lack of effect of low purine diets [28,36], support the contention that diet has a minimal direct effect on 8OH2'dG levels. The low levels in feces, and the observation of loss of 8OH2'dG in food pools under conditions mimicking digestion, may reflect a mechanism supporting these prior findings.

The comparison of mean analyte levels and artifacts among different studies and procedures is traditional in methods discussions, but primarily only serves to estab-

lish consensus on a general range for values. There is clearly a major impact of analytic methodology on the use and interpretation of 8OH2'dG results. Even for well-established analyses of 8OH2'dG in DNA digests, recent European Standards Committee on Oxidative DNA Damage interlaboratory studies have shown several orders of magnitude rather than percentage differences among methods and laboratories [38].

Studies of the biologic relevance of 8OH2'dG to disorders or intervention are impacted by three factors. First, sampling artifacts and disorder-related interferences can affect both preparative and analytic steps in an assay. Disorders clearly increase analytic complexity and each disorder category effectively constitutes a different analytic matrix. Second, there has been no consistent convention for describing the precision and accuracy of analytic methods. Short-term and long-term precision can vary significantly, and ultimately, the precision of every study data set should be evaluated independently. Third, a large number of samples are required to establish a categorical difference in view of the variability of 8OH2'dG levels among control individuals.

Because of these issues, the design of this study focused, after establishing the method, on reduction of possible artifacts and on controlling, documenting, and testing the precision and accuracy of 8OH2'dG measurements over an extended period under routine conditions. The evaluative studies of the analytic system confirmed that there were no instrumentally introduced artifacts. There was quantitative recovery of 8OH2'dG and no evidence of production of 8OH2'dG from 2'dG under extreme settings of the conditions. This was reflected throughout the 1-year evaluation by the quantitative recovery of 8OH2'dG from the 427 spiked samples and pools of all of the matrices studied.

The variation of system conditions confirmed that the same values were obtained for urine pools from different disorders under multiple conditions, which strongly indicates qualitative certainty for 8OH2'dG in the most complex samples available. The qualitative assurance measures for routine assay derived from these studies were stringent. For a compound to give an undetected interference for 8OH2'dG it must elute at the same time from a C8 column, have the same retention time on a carbon column, and be displaced from the carbon column by adenosine at the same rate. Then it must have the identical retention time on a C18 column in a different mobile phase (i.e., give no change in retention time or $W_{1/2}$ measures), and have identical electrochemical characteristics (i.e., show no change in the T_4/T_3 ratio).

The quality control data in Tables 2-4 demonstrate a number of points related to the hierarchy of errors in a method. The precision of the control standards of approximately $\pm 2-3\%$ rsd over the 1-year interval is sta-

tistically significantly greater than the $\pm 0.51-1.14\%$ rsd from repetitive injections of the standards in evaluation studies. The difference reflects the imprecision introduced by multiple variables affecting operation of the system over a 1-year vs. a 1-d time period. The values for the regression standards run over 1 year also show increased rsd over 1-d studies. However, both levels of rsd indicate that instrumental stability is a minor factor in the precision of the assays. The regression standard values show that the assumption of linearity made from evaluation studies is valid over the 1-year evaluation period. The reduction in precision as a function of the total quantity of 8OH2'dG loaded on the column is consistent with the precision vs. concentration characteristic found in evaluation studies.

These observations indicate that sample preparation and sample composition were the major variables contributing to rsd. The magnitude of the effect of sample composition for urine is small, but evident, as seen from the rsd of duplicate urine pairs ($\pm 4.19\%$ rsd), which is greater than that of regression standards calculated at the same level ($\pm 3.08\%$ rsd). In the design of the analytic sequences, duplicate samples and regression standards were both independent of long-term drift effects, and should have had the same precision. The preparative process of diluting the urine samples 1:1 with buffer should with reasonable care introduce only a $\pm 0.5\%$ error. Thus, over the range of disorders studied, there was a contribution of $\pm 2.8\%$ rsd from the urine matrix, most probably in the estimation of baseline in real samples vs. standards.

The rsd of the urine pools, which reflects all variations in factors of preparative differences, drift and response over a 1-year period was, as expected, greater than that of duplicates that reflect only the within-sequence variation. The magnitude of the difference would indicate that the agreement of the means of a large set of samples would typically vary by $\pm 3\%$ at any time interval during a 1-year period. The recovery data predict that any spiked sample over basal urine should be recovered with 100% accuracy with precision of approximately $\pm 5\%$ rsd. For the objective of supporting long-term studies of 8OH2'dG as a risk factor or therapeutic monitoring tool, the data would indicate that individual values differing by approximately $\pm 8\%$ in short-term studies and approximately $\pm 12\%$ in studies extending for 1 year could be ascribed with 95% confidence to biologic and not analytic differences.

In the absence of structured interlaboratory proficiency programs, blind internal and external testing of methodology are the penultimate tool for assessing the validity of QC protocols. The internal blind testing with 50 duplicate urine pairs and the eight-sample set provided by NIOSH had precision equivalent to that pre-

dicted. The larger set of blind accuracy and precision tests provided by RJ Reynolds Tobacco Inc., although also confirming predictions, raised critical points about analytic sequences and study design.

The recovery data from the blind spiked urine samples indicated a precision of less than $\pm 2\%$ rsd. The better-than-predicted performance reflected three factors: in retrospect by chance the samples were analyzed over a 3-d interval in adjacent sequences; because they were spikes into the same pool they had identical baseline characteristics; and again by chance, control standard precision during the particular set of runs was rsd $\pm 0.93\%$. In a study design where small changes are important, analytic error can be reduced by choosing the sequence in which samples are run.

The discrepancy in the rsd values from the entire data set of 54 blind subaliquoted stored urine samples and the rsd predicted by internal QC, without elimination of two outlier samples raises several issues. For both statistical and observational reasons, the two samples contributing the majority of the imprecision can be legitimately rejected for method evaluation. Doing this puts the blind precision in agreement with the predicted long-term precision of the method, which is comforting to the analyst. Nevertheless, the precision of the data set as opposed to the method precision must still be taken as that measured by the blind samples as an entire group. The internal and external blind studies of urine assay precision confirmed that predicted by the QC measures. However, they also demonstrated that no global estimate of precision for a set of data could be made from laboratory studies alone, and showed the need for control of analytic data on a study-specific basis.

Given analytic methods of sufficient precision and discrimination, sample acquisition, subaliquoting, and storage can have a greater effect on the fidelity of data than preparation and assay. In the simplest example discussed here, because of the contribution of particulate material to variation in results, stored bulk urine samples must be thoroughly homogenized before subaliquoting for 8OH2'dG assays. If stored samples are subaliquoted without homogenization or filtered before dilution, our studies indicate that a systematic error of up to a factor of two could be introduced into a study. Ideally, sample sets of any matrix should be subaliquoted at the time of acquisition and only taken from storage for a specific assay.

As shown in Table 2, the methodology for filter paper urine samples has sufficient precision for screening of neonatal populations. The higher rsd values for filter paper urine samples are consistent with both the lower levels of 8OH2'dG in the filter paper extracts and with the increased error introduced by the precision of the creatinine divisor. During the time interval of the studies,

precision of blind duplicate samples submitted externally for creatine assay for bulk urines was $\pm 4.02\%$ rsd, and internally for filterpaper extracts precision was $\pm 6.14\%$ rsd.

As shown in Table 3, recoveries of various preparation protocols for different matrices were $>95\%$, and the precision of the pools and duplicates was consistent with both the levels of 8OH2'dG and the variation in recovery. The precision of the control, regression, and recovery standards indicates no systematic drift caused by the various matrices with SPE concentration. The slightly higher rsd and drift in level of control standards with precipitation concentration methods reflects the variations in gate 1 caused by these preparations. As shown in Table 4, for DNA digests, the precision of the 2'dG measurements was equivalent to that of the 8OH2'dG, and the precision of the mole ratios in pools was consistent with the combined assay errors. The principal advantage of the system for DNA digest assays was elimination of sample induced baseline artifacts in the measurement of 8OH2'dG. This allowed simultaneous measurements of 8OH2'dG at levels of 40 pg/ml and 2'dG at 400 $\mu\text{g/ml}$ with approximately 7% rsd, and consequently, determinations of mole ratios of 8OH2'dG/2'dG as low as 1×10^{-7} .

There are several study design possibilities that arise from the current evaluation. The quality assurance data abstracted over the 1-year evaluation demonstrate that the system and analytic methodology are suitable for long-term, high-volume epidemiologic studies of urinary or plasma 8OH2'dG as a risk factor. The high level of stability to room temperature conditions in bulk and filter paper urine and plasma make 8OH2'dG in these matrices highly attractive as a biologic process marker from an operational standpoint. With reasonable care in acquisition, storage, subaliquoting, and preparation procedures, both freshly acquired and stored samples are suitable for such studies.

The applicability to a range of disorders offers utility in long-term evaluations of therapeutic interventions, as well as studies of categorical differences in disease. Because 8OH2'dG output and 8OH2'dG/creatinine ratios are quite stable in an individual over time [20], it would be possible to use a relatively small number of serial spot urine samples to ascribe changes as small as $\pm 4\%$ in an individual's mean levels to biologic rather than analytic factors resulting from a particular insult or intervention. Currently, it is a matter of speculation whether changes of this magnitude could have significant biologic or therapeutic implications.

It is also critical to realize that 8OH2'dG measurements in a given study could be more precise than the creatinine or urine output volume per unit time measurements, used as divisors to assess excretion rate. During

this study, by the same level of blind internal and external testing, the creatinine assay precision was equivalent to the 8OH2'dG precision. For volume/time measurements, the data from this study would indicate that a time error of 30 min and a volume error of 30 ml in a 24-h urine collection would in the worst case contribute twice as much error to output assessment as the 8OH2'dG assay. To utilize the assay capability fully, considerable care should be taken to control both established correlative assays and/or sample acquisition precision.

Matrices other than plasma and urine have not been assayed over sufficient time or in sufficient numbers to evaluate their utility for long-term studies; however, the rsd of the methods for other matrices is small relative to biologic variability. The ability to measure 8OH2'dG in the majority of accessible body reservoirs, in foods, and various model systems should allow the configuration of studies to further define compartmental relationships and the routes, rates, and reservoirs of production, intake, and excretion. The ability to measure 8OH2'dG levels in brain and muscle microdialysates, tissues, and culture matrix may allow better estimates to be made of the mechanisms of production, excision, and removal from specific organs and the effect of apoptosis on observed levels.

Although it is not in the scope of this article to comment quantitatively on these issues, a few observations are indicated by the simple mean levels of 8OH2'dG. The observation of production in *C. elegans* culture, where cell number is conserved through the life cycle, would argue that, at least in simple organisms, apoptosis does not play a major role in 8OH2'dG production. The low levels in plasma indicate that 8OH2'dG is cleared rapidly from the body. The even lower levels in CSF and dialysates suggest the possibility of active clearance mechanisms from specific sites of production. The levels in sweat and saliva indicate that in some extreme situations, such as kidney failure or extensive exercise, these compartments may have to be considered in estimation of production and excretion rates.

Conclusions

Routine high-precision analytic methods for 8OH2'dG based on LCEC column-switching with carbon columns offer the possibility of evaluating this marker of oxidative DNA damage as a practical clinical, therapeutic monitoring, and epidemiologic tool.

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Research report

Increased nitrotyrosine immunoreactivity in substantia nigra neurons in MPTP treated baboons is blocked by inhibition of neuronal nitric oxide synthase

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Research report

Increased nitrotyrosine immunoreactivity in substantia nigra neurons in MPTP treated baboons is blocked by inhibition of neuronal nitric oxide synthase

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Abstract

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) produces clinical, biochemical and neuropathologic changes reminiscent of those which occur in idiopathic Parkinson's disease. 7-Nitroindazole (7-NI) is a relatively selective inhibitor of the neuronal isoform of nitric oxide synthase. We previously demonstrated that administration of 7-NI is effective in blocking MPTP toxicity in both mice and baboons. This was suggested to be due to inhibition of the generation of peroxynitrite which can nitrate tyrosines. In the present study we found increased 3-nitrotyrosine immunoreactivity in the substantia nigra of MPTP treated baboons, which was blocked by coadministration of 7-NI. These findings provide further evidence that peroxynitrite may play a role in MPTP induced parkinsonism in baboons. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: MPTP; 3-Nitrotyrosine; Parkinson's disease; Nitric oxide; Peroxynitrite

1. Introduction

In human and nonhuman primates MPTP produces clinical, biochemical and neuropathologic changes similar to those which occur in idiopathic Parkinson's disease [6]. It produces motor abnormalities consisting of bradykinesia, increased muscle tone and a characteristic resting tremor. Furthermore it induces marked depletion of dopamine concentrations as well as a loss of tyrosine hydroxylase positive neurons in the substantia nigra.

The pathogenesis of neuronal degeneration following MPTP administration has been intensively investigated. The neurotoxic effects of MPTP are thought to be mediated by its metabolite 1-methyl-4-phenylpyridinium (MPP⁺) which is produced by the oxidation of MPTP by monoamine oxidase B [24]. MPP⁺ is selectively taken up

by the high affinity dopamine and noradrenaline uptake systems, and is subsequently accumulated within mitochondria of dopaminergic neurons. There it disrupts oxidative phosphorylation by inhibiting complex I of the electron transport chain [12]. This can lead to a number of deleterious effects on cellular function. These include impaired intracellular calcium buffering as well as generation of free radicals from mitochondria and activation of neuronal nitric oxide synthase, a calmodulin dependent enzyme [1]. The generation of the free radical nitric oxide (NO[•]) followed by the production of peroxynitrite has been implicated in cell death [2,4,9,11].

We previously showed that 7-nitroindazole (7-NI) is a relatively selective inhibitor of the neuronal isoform of nitric oxide synthase and can produce marked protection against MPTP induced parkinsonism in baboons [14]. It protected against both dopamine depletions as well as loss of substantia nigra tyrosine hydroxylase positive neurons, motor deficits and frontal type cognitive dysfunction. In the present study we examined whether 7-NI also protected against increases in 3-nitrotyrosine immunoreactiv-

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ity in the substantia nigra, which is thought to be a relatively specific marker for peroxynitrite mediated neurotoxicity [5,15].

2. Materials and methods

Twelve male *Papio Anubis* baboons were used in these experiments as previously reported [14]. The animals were distributed into four groups: control animals, $n = 2$; MPTP treated animals, $n = 4$; 7-NI injections, $n = 2$; and MPTP/7-NI treated animals, $n = 4$. An acute model of MPTP intoxication was used. Daily intramuscular MPTP

injections (Sigma, as solutions of the hydrochloride salt) were given for 7 days according to the following protocol: 0.4 mg/kg in two daily injections at 1100 and 1800 h from day 1 to day 6, and finally 0.27 mg/kg injection at 1100 h on day 7. 7-Nitroindazole (Lancaster, Morecambe, England) was administered subcutaneously as a 25 mg/kg suspension in peanut oil to 6 animals (four in the MPTP/7-NI group and two in the 7-NI group) as two daily injections at 0900 and 2100 h, with the first injection given two hours before the MPTP injection in the MPTP/7-NI group. The 7-NI treatment was continued until day 10 upon which the animals were sacrificed.

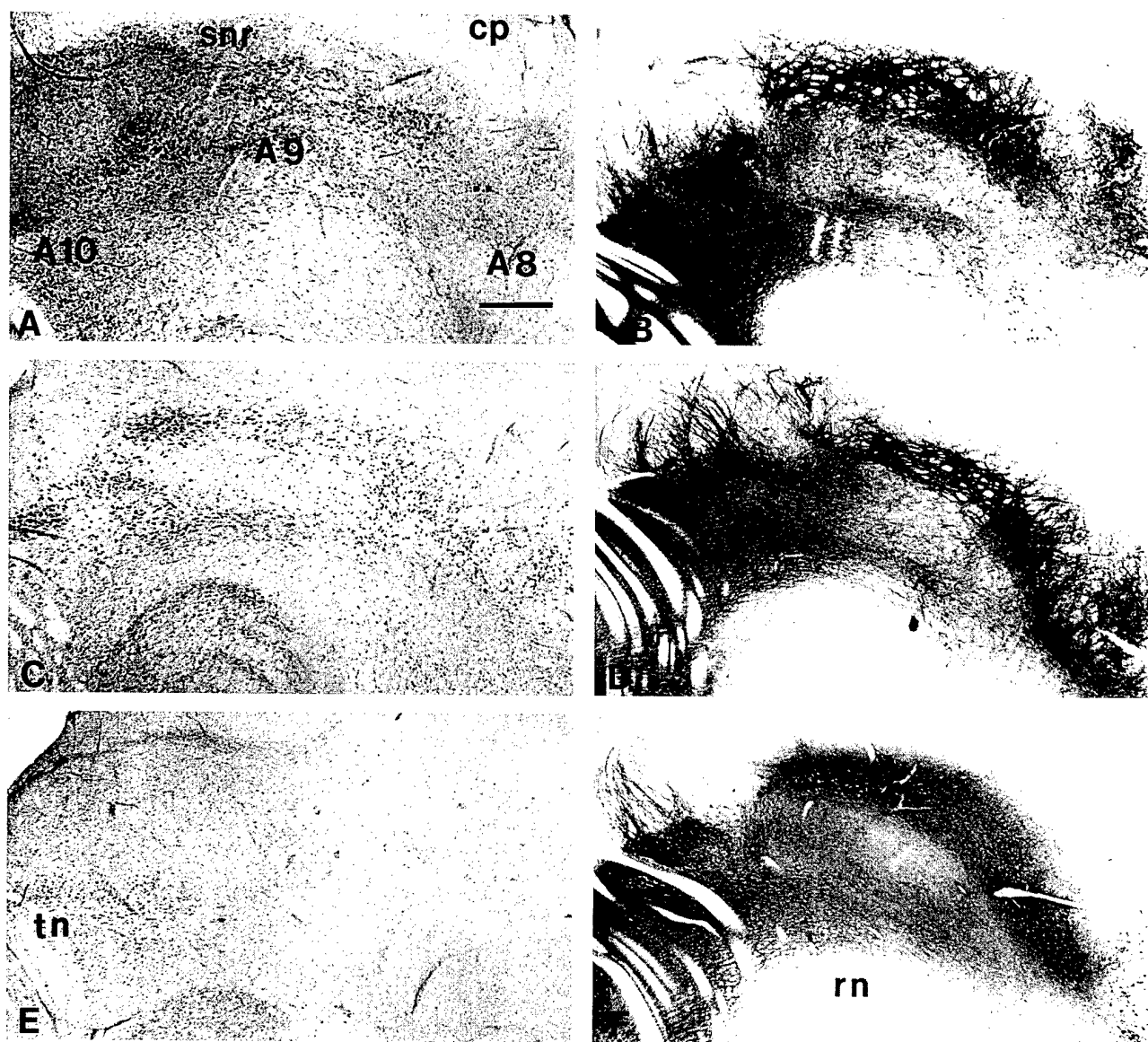


Fig. 1. Photomicrographs of the substantia nigra at the level of the red nucleus (RN) and exit of the third nerve (tn) of Nissl stained and tyrosine hydroxylase (TH) stained sections from saline control- (A and B), MPTP/7-NI (C and D), and MPTP-treated animals (E and F). There is a significant loss of both Nissl and TH-positive neurons with concomitant astrogliosis in the MPTP treated animal in comparison to the saline control animal. Neurons represented by Nissl staining and TH immunoreactivity are relatively spared in the MPTP/7-NI treated animal. Magnification bar in A represents 1 mm. snr: substantia nigra reticulata, cp: cerebral peduncle.

Animals were sacrificed under chemical restraint (ketamine 10 mg/kg) using deep pentobarbital anesthesia (120 mg/kg) and the brains were rapidly removed. The

brainstem of each animal was immersion fixed for 48 h in cold (4°C) paraformaldehyde 2% sodium-*m*-periodate lysine, cryoprotected in a graded series of 5, 10, and 20%

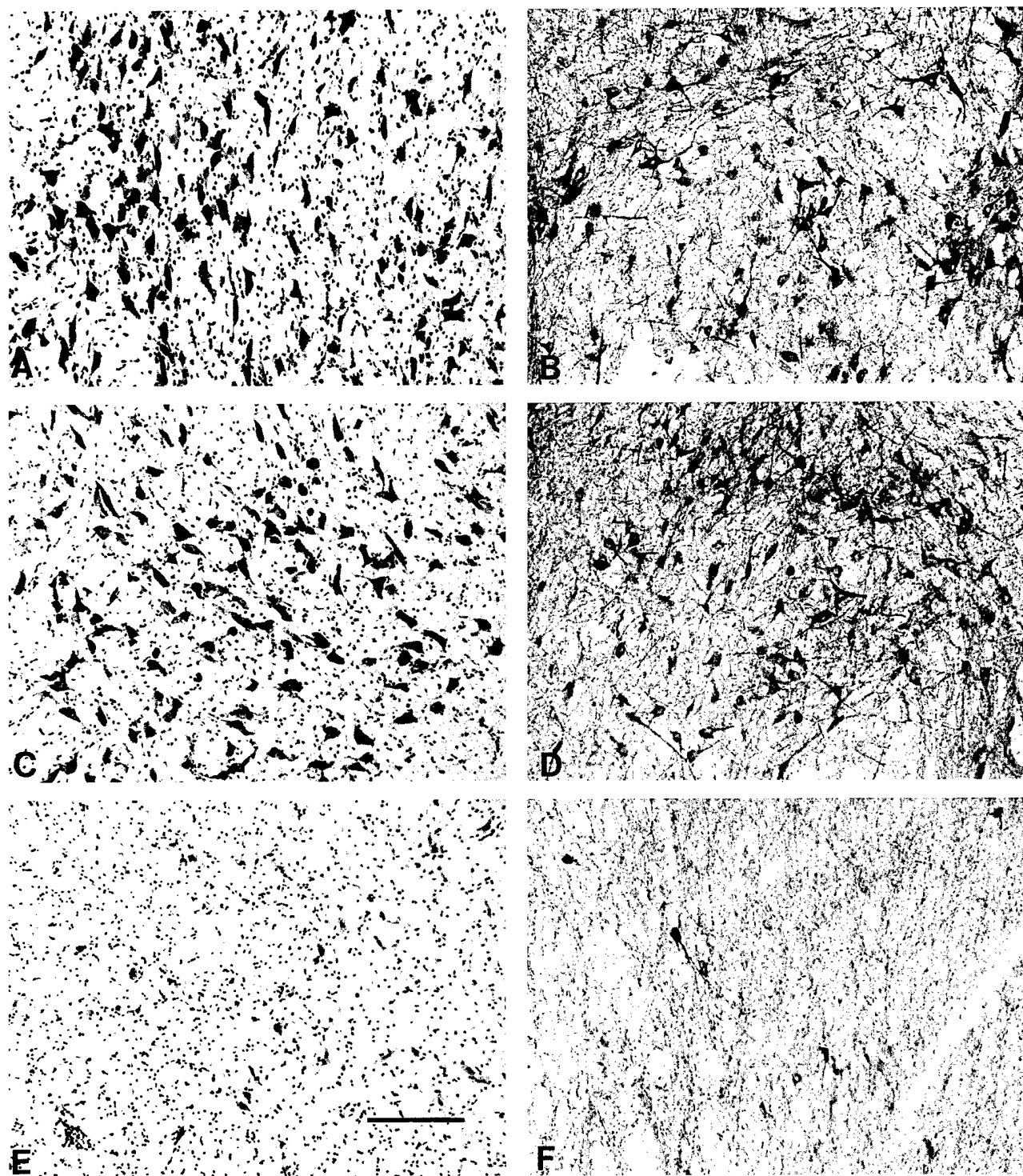


Fig. 2. Photomicrographs of Nissl staining and tyrosine hydroxylase (TH) immunoreactivity in the substantia nigra (A9) from Fig. 3 in saline- (A and B), MPTP/7-NI (C and D), and MPTP- (E and F) treated animals, respectively. Note the marked Nissl and TH-positive neuronal loss using MPTP alone (E and F), in comparison to MPTP/7-NI and controls. Neuronal soma are atrophic with concomitant dendritic arbor reduction. There is some preservation of axonal arbors, in the form of beaded rosettes, within the substantia nigra compacta (A9). This most likely is the consequence of preserved projections from lateral (10) and medial (A8) areas. Neurons are relatively preserved in MPTP/7-NI treated animals (C and D) and are without dysmorphic changes. Magnification bar in E = 200 μ m and is the same in A, B, C, D, and F.

phosphate-buffered glycerol, and subsequently frozen-cut and serially-sectioned coronally at 50 μm and stained for routine cell identification using cresyl violet (Nissl stain). Immunocytochemistry for tyrosine hydroxylase (Eugene Tech International, 1:1000 dilution) and 3-nitrotyrosine (Upstate Biotechnology, 1:500 dilution) immunoreactivity was carried out as previously described [2,11,14].

Tissue sections were preincubated in absolute methanol-0.3% hydrogen peroxide solution for 30 min, washed ($3\times$) in phosphate buffered saline (PBS) (pH 7.4) 10 mins each, placed in 10% normal goat serum (Gibco Labs) for 1 h, incubated free floating or on slide in primary antiserum at room temperature for 12–18 h (dilution of primary antisera above included 0.3% Triton X-100 and 10% normal goat serum), washed ($3\times$) in PBS for 10 min each, placed in periodate-conjugated goat anti-rabbit IgG (1:300 in PBS) (Boehringer-Mannheim), washed ($3\times$) in PBS 10 mins each, and reacted with 3,3'-diaminobenzidine HCl (1 mg/ml) in Tris-HCl buffer with 0.005% hydrogen peroxide.

Specificity for the antisera used in this study was examined in each immunohistochemical experiment to assist with interpretation of the results. This was accomplished by preabsorption with excess target proteins and by omission of the primary antibody to determine the amount of background generated from the detection assay. The nitrotyrosine antibody was tested by preadsorption of dilute primary antisera with an excess of appropriate nitrotyrosine protein (Sigma, 4 $\mu\text{g}/\text{ml}$) for 6 h at room temperature prior to incubation.

All animals were housed individually in standard primate cages with free access to water and food. The studies were completed in accordance with the Animal Care and

Use of Laboratory Animals presented by the National Institutes of Health.

3. Results

Marked Nissl and tyrosine hydroxylase immunopositive neuronal loss was present throughout the substantia nigra of MPTP-treated animals and was consistent with previous reports (Figs. 1 and 2). Neuronal loss was most prominent in the A9 area, with relative preservation of the ventral tegmental area (A10) and the lateral retrorubral area (A8) of the substantia nigra. While some neuronal loss was present in the A9 area of MPTP/7-NI-treated animals, it was not significant in comparison to MPTP treatment alone. There was relative sparing of neurons within the substantia nigra in MPTP/7-NI-treated animals. Previously reported Nissl and tyrosine hydroxylase immunopositive neuronal cell counts from this experiment verified these observations [14].

Neuronal 3-nitrotyrosine immunoreactivity was markedly increased in remaining substantia nigra neurons in MPTP-treated animals in comparison to MPTP/7-NI-treated and control animals (Fig. 3). Although the most intense 3-nitrotyrosine immunoreactivity was present within neurons in MPTP-treated animals, 3-nitrotyrosine immunoreactivity was also present throughout the neuropil, including the vasculature and other tissue elements. In contrast, while there was some variability in the expression of 3-nitrotyrosine immunoreactivity in MPTP/7-NI-treated animals, neuronal and neuropil localization of 3-nitrotyrosine was only light or faintly positive in the MPTP/7-NI-treated animals, with little or no 3-nitrotyro-

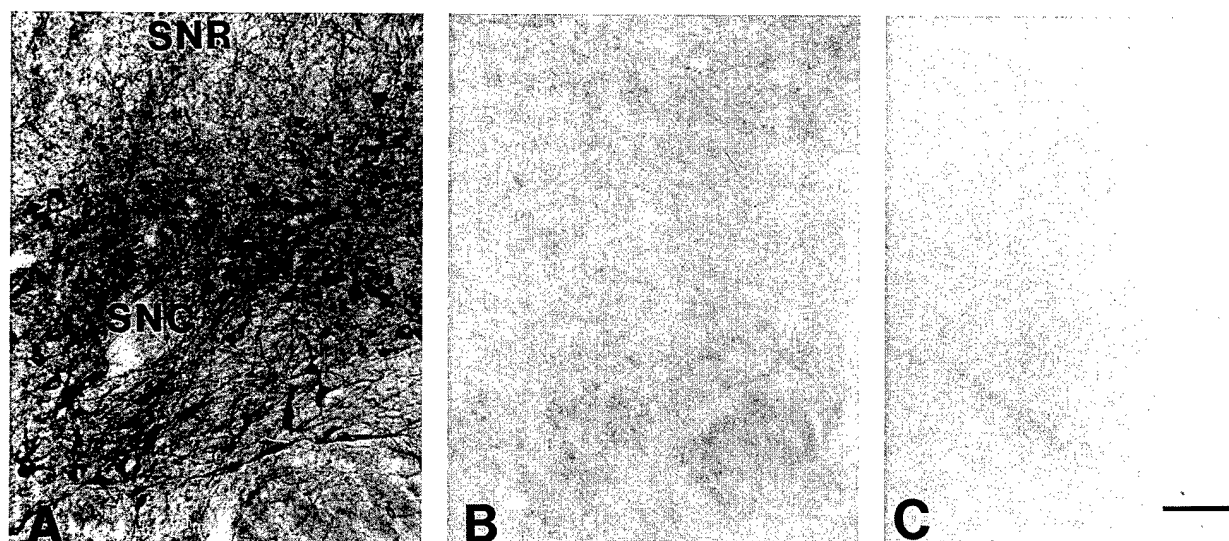


Fig. 3. Photomicrographs of 3-nitrotyrosine immunoreactivity in the substantia nigra of MPTP- (A), MPTP/7-NI (B), and saline control- (C) treated animals. Intense 3-nitrotyrosine immunoreactivity was present in remaining neurons of the substantia nigra compacta (SNC) with moderate 3-nitrotyrosine immunoreactivity in the neuropil. In the MPTP/7-NI specimens, 3-nitrotyrosine immunoreactivity was slightly increased in cells and the neuropil above the saline control. Magnification bar in C represents 200 μm and is the same in A and B. SNR: substantia nigra reticulata.

sine immunoreactivity in control animals (Fig. 3). The substantia nigra from animals treated with 7-NI alone looked no different than control animals. PreadSORption of dilute primary nitrotyrosine antisera with an excess of appropriate nitrotyrosine protein (Sigma, 4 µg/ml) for 6 h at room temperature prior to incubation resulted in no immunohistochemical detection within the tissue specimens.

4. Discussion

The mechanism of cell death following MPTP administration has been intensively investigated since the initial reports that it could produce parkinsonism in both man and animals. Evidence for oxidative damage in the pathogenesis of MPTP neurotoxicity comes from observations that MPTP toxicity is significantly attenuated in transgenic mice overexpressing superoxide dismutase [20]. We previously showed that the relatively selective inhibitor of neuronal nitric oxide synthase, 7-nitroindazole, produces dose dependent protection against MPTP induced dopamine depletion and increases in 3-nitrotyrosine in mice [22]. This finding was confirmed by Przedborski et al. [19] who demonstrated that 7-nitroindazole protected against MPTP toxicity as well as MPTP induced loss of tyrosine hydroxylase positive neurons. Furthermore, these authors demonstrated that mice with a knockout of the neuronal isoform of nitric oxide synthase were relatively resistant to MPTP neurotoxicity. Similarly we found that mice with a knockout of the neuronal isoform of nitric oxide synthase are resistant to MPP⁺ induced loss of substantia nigra neurons [17]. S-methylthiocitrulline is another relatively selective neuronal nitric oxide synthase inhibitor that also protects against MPTP neurotoxicity in mice [18]. 7-nitroindazole when coadministered with MPTP produced marked neuroprotective effects against the neurochemical, histologic, and behavioral deficits associated with MPTP toxicity in baboons [14]. Coadministration of 7-nitroindazole protected against 94–98% depletions of dopamine in both the caudate nucleus as well as the putamen. 7-Nitroindazole however has been reported to also be a monoamine oxidase B inhibitor in vitro which could contribute to its neuroprotective effects [8,10]. It has also been reported that L-nitroarginine, a nonspecific NOS inhibitor does not protect against MPTP toxicity in marmosets [16].

The findings implicate peroxynitrite in the pathogenesis of MPTP neurotoxicity. Peroxynitrite is formed by the interaction of nitric oxide with superoxide radical to form peroxynitrite, which occurs at a rate of 6.7×10^{-9} M/s and does not require transition metals [4]. It can diffuse over several cell diameters where it can oxidize lipids, proteins and DNA by a 'hydroxyl radical-like' mechanism. 3-Nitrotyrosine appears to be a relatively specific marker of peroxynitrite mediated nitration of proteins [3,15]. The source of NO in the substantia nigra is unclear since one

report did not find NOS immunoreactive neurons in the substantia nigra [7], however we found a small population of immunoreactive neurons [17].

In the present study we therefore examined whether 3-nitrotyrosine immunoreactivity shows increased expression in substantia nigra neurons after systemic administration of MPTP in baboons. An increase in 3-nitrotyrosine immunoreactivity was demonstrated in monkeys which received MPTP as compared to normal controls. Furthermore the increased nitrotyrosine immunoreactivity was attenuated by pretreatment with 7-nitroindazole. These findings therefore provide further evidence implicating peroxynitrite in the pathogenesis of neuronal cell death mediated by MPTP.

There is also evidence that peroxynitrite may play a role in Parkinson's disease. An increase in cerebrospinal fluid nitrite concentrations has been reported in Parkinson's [21] and NO[•] radicals have been detected in PD substantia nigra [23]. Lastly the core of Lewy bodies in Parkinson's disease has been shown to be immunoreactive for nitrotyrosine [13]. There is therefore evidence implicating protein nitration in both MPTP and PD, which suggests that inhibition of neuronal nitric oxide synthase may be a useful therapeutic strategy for PD.

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INCREASING evidence implicates apoptosis as a major mechanism of cell death in neurodegenerative diseases. Recent evidence has demonstrated that chronic administration of MPTP can lead to apoptotic cell death. In the present study we examined whether transgenic mice expressing a dominant negative inhibitor of interleukin-1 β converting enzyme (ICE) are resistant to MPTP induced neurotoxicity. MPTP resulted in a significant depletion of dopamine, DOPAC and HVA in littermate control mice which were completely inhibited in the mutant interleukin-1 β converting enzyme mice. There was also significant protection against MPTP-induced depletion of tyrosine hydroxylase-immunoreactive neurons. There was no alteration in MPTP uptake or metabolism. These results provide further evidence that apoptotic cell death as well as ICE may play an important role in the neurotoxicity of MPTP. *NeuroReport* 10:635-638 © 1999 Lippincott Williams & Wilkins.

Transgenic mice expressing a dominant negative mutant interleukin-1 β converting enzyme show resistance to MPTP neurotoxicity

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Key words: Apoptosis; Caspases; Dopamine; MPTP; Parkinson's disease

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Introduction

There is increasing evidence implicating apoptosis as a major mechanism of cell death in a variety of neurodegenerative diseases. Evidence for this in Parkinson's disease (PD) at present is controversial. One study using *in situ* end-labeling demonstrated DNA damage whereas another did not [1,2]. A recent study using electron microscopy found morphological evidence of apoptosis in the substantia nigra of a brain from a PD patient [3]. Furthermore an increase in Bcl-2 protein was found in the caudate nucleus and putamen of PD patients, and this also appears to occur in patients with incidental Lewy body disease [2,4].

The ced 3/ICE cell death family, also known as caspases, are important mediators of apoptotic cell death. The caspase family now consists of at least 12 members which can be classified into three classes

based on the homology with each other: (1) the interleukin converting enzyme (ICE) subfamily including ICE (caspase-1, ICE REL2-II, ICE REL3-III and ICH-3), (2) a CPP32 subfamily including CPP32 (caspase-3, MCH-2, MCH-3, FLICE and MCH-4) and (3) ICH-1 subfamily including ICH-1 and MCH-6 [5]. All caspases are synthesized as inactive enzymes. They are activated by proteolytic cleavage at aspartate-X sites and in turn cleave at aspartate-X sites. Their active sites contain a conserved pentapeptide QACXG. Caspases may act in protease cascades which are activated by specific signals. A critical role of ICE-like proteases and apoptosis has been well established. ICE knockout mutant mice generated by gene targeting techniques were found to be only partially defective in apoptosis induced by anti-fas antibody [6]. The neurons from ICE-KO mice showed resistance to trophic factor-mediated apoptosis [7,8].

ICE-KO mice are also resistant to MCA occlusion-mediated infarcts. Other studies show elevated levels of mature interleukin-1 β following apoptotic cell death indicating activation of ICE in apoptosis [9,10].

Recently transgenic mice were generated in which cysteine in the active site of ICE was replaced by glycine (C285G) [7]. The cysteine residue in the active site is required for the IL-1 β convertase and the auto-processing activity of ICE. In the present experiments we utilized this transgenic mouse model expressing ICE C285G mutant under the control of neuronal enolase promoter (NSE-M172). We examined the susceptibility of these mice to MPTP-induced neurotoxicity.

Materials and Methods

MPTP neurotoxicity was evaluated in littermate wild-type control in C285G ICE mice. These mice were initially developed as described previously [7]. The mice show no microscopic or gross abnormalities of the central nervous system. MPTP (Research Biochemicals, Wayland, MA USA), dissolved in water with the pH adjusted to 7.4, was administered at a dose of 15 mg/kg every 2 h for four doses. Controls received phosphate buffered saline. Ten animals were examined in each group. Animals were sacrificed at 1 week and the striata were dissected and placed in 0.1 M perchloric acid. Samples were sonicated, centrifuged and an aliquot was injected for measurements of dopamine, DOPAC and HVA by HPLC with electrochemical detection as described previously [11]. To determine whether MPTP uptake or metabolism was altered MPTP was administered at a dose of 30 mg/kg every 2 h for two doses and mice were then sacrificed at 1 h. The striata were dissected. 1-Methyl-4-phenylpyridinium (MPP⁺) levels were quantified by HPLC with u.v. detection at 295 nm. Samples were sonicated in 0.1 M perchloric acid and an aliquot of supernatant was injected onto a Brownlee aquapore X03-224 cation exchange column (Rainin, Woburn, MA USA) and diluted with 90% 0.1 M acetic acid, 75 mM triethylamine HCl (pH 2.3) and 10% acetonitrile.

Histopathological evaluation of eight wild-type littermate control, eight wild-type littermate control/MPTP-treated, and eight C285G ICE/MPTP-treated mice was performed. These mice were deeply anesthetized and then transcardially perfused with 4% buffered paraformaldehyde. The brains were removed, post-fixed with the perfusate for 2 h, and cryoprotected in a graded series of 10% and 20% glycerol/2% DMSO solution. The brain specimens were cut on a cryostat at 50 μ m and stained for Nissl

(cresyl violet) and immunohistochemically for tyrosine hydroxylase (TH; TH antisera, 1:1000 dilution; Eugene Tech International) using a previously reported conjugated second antibody method [12]. Midbrain sections through both the left and right substantia nigra from bregma levels -3.08 mm to -3.16 mm and intra-aural levels 0.72 mm to 0.64 mm in each of the wild-type littermate control and eight C285G ICE mice were analysed by microscopic-videocapture. Neuronal counts of TH-positive neurons within the entire substantia nigra pars compacta and pars reticulata from 2-3 sections of each case were computed using Neurolucida (Microbright-field) image analysis software. Correction for tissue section thickness was made in all specimens. The data are expressed as the mean \pm s.e.m. Statistical comparisons were made by one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference *post hoc* test (PLSD).

Results

As shown in Fig. 1 there were no significant differences in striatal dopamine, DOPAC or HVA concentrations following administration of phosphate buffered saline in wild-type littermate control and C285G ICE mice. Following administration of MPTP there was a significant reduction of dopamine, DOPAC and HVA concentrations in the littermate controls. In contrast in the C285G ICE mutant mice there were no significant depletions of dopamine, DOPAC or HVA. As compared to the littermate control mice treated with MPTP the concentrations of dopamine, DOPAC and HVA were significantly increased.

We examined MPP⁺ levels to be certain that there was no alteration in MPTP uptake or metabolism. MPP⁺ levels were not significantly different in the littermate control and the C285G ICE mice (50.6 ± 7.6 and 45.7 ± 3.9 ng/mg protein, respectively).

Histological examination showed that in the MPTP-treated littermate control mice there were selective bilateral lesions within the substantia nigra, with no pathological alterations in the C285G mice. There was no significant loss of neurons in the C285G/MPTP-treated animals, while the severity of neuronal loss within the substantia nigra in MPTP-treated littermate control animals was marked, especially within the medial aspect (Fig. 2). Neuronal counts from TH-immunostained sections within the substantia nigra confirmed the neuronal loss in the control/MPTP-treated mice in contrast to control untreated mice and the C285G/MPTP-treated mice (control untreated mice 126 ± 4 ; control/MPTP-treated mice 38 ± 7 ; C285G mice 120 ± 5 , $P < 0.01$).

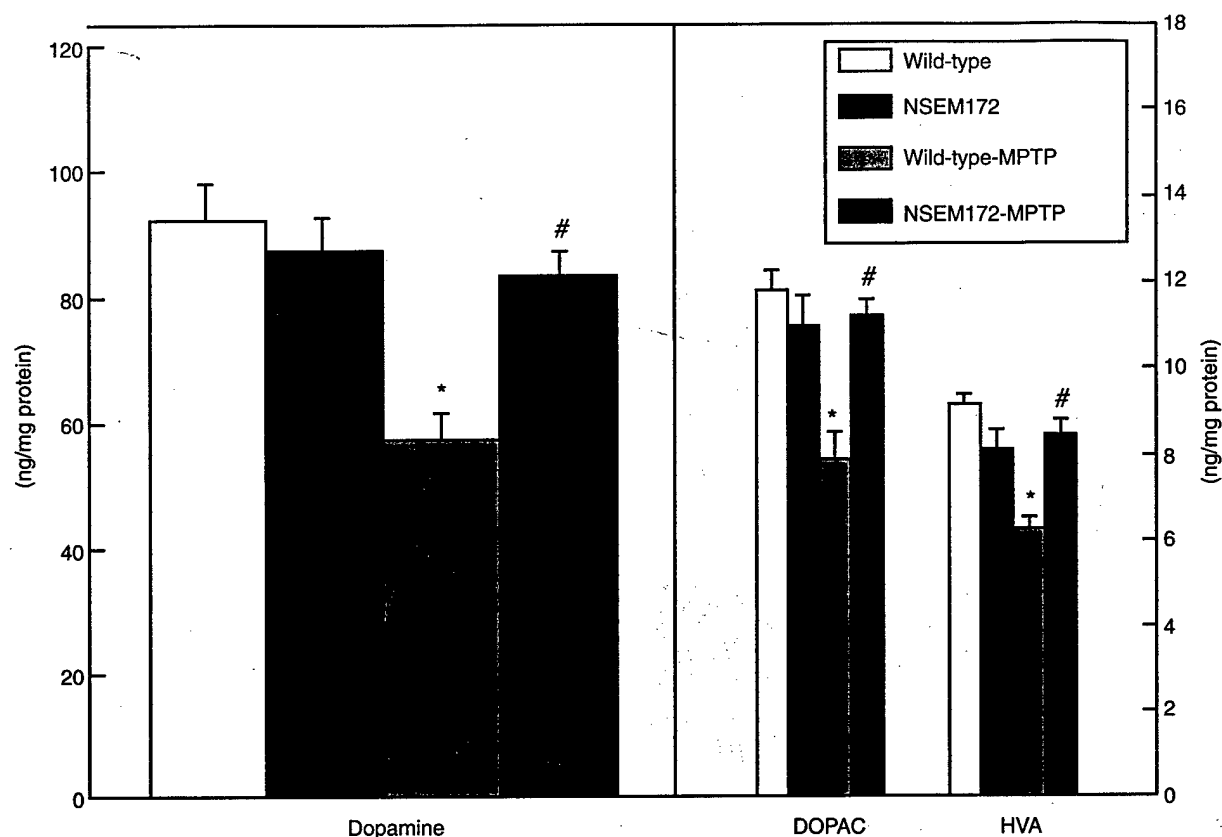


FIG. 1. The effects of MPTP (15 mg/kg, i.p. $\times 4$) on striatal dopamine, DOPAC and HVA in wild-type littermate control and C285G ICE (NSE-M172) transgenic mice. * $P < 0.001$ compared with control levels. # $P < 0.001$ compared with MPTP-treated control mice. There was no statistical difference between untreated control mice and MPTP-treated C285G ICE transgenic mice.

Discussion

Substantial evidence implicates apoptotic cell death in a number of disease processes [13]. Recently it was demonstrated that chronic administration of MPTP was associated with evidence of apoptotic cell death in the substantia nigra [14]. If this is indeed the case one might expect that mice which have a deficiency in caspase activation might show resistance to MPTP neurotoxicity. In the present experiments we utilized a transgenic mouse strain expressing a dominant negative ICE inhibitor in the brain [7]. Developmental apoptosis in these mice appears not to be inhibited as evidenced by normal numbers of neurons in the facial motor nucleus compared with wild-type littermates. Expression of the ICE C285G mutant is a dominant-negative inhibitor of ICE that can inhibit processing of prointerleukin-1 β by ICE *in vivo*. Expression of mutant ICE C285G in dorsal root ganglia neurons either by microinjection or in transgenic mice inhibits trophic factor withdrawal-induced apoptosis [7]. Following systemic injection of lipopolysaccharide which induces release of mature interleukin-1 β

in wild-type mice, the whole brain lysates of mutant ICE C285G transgenic mice contained 75% less mature interleukin-1 β than did lipopolysaccharide-injected wild-type mice. Similarly there was no detectable mature interleukin-1 β in brain lysates following i.p. injection of saline in the mutant ICE C285G mice, whereas this was detected in wild-type mice, since ICE is required for prointerleukin-1 β processing [6,15]. This evidence suggests that mutant ICE C285G can act as an effective inhibitor of prointerleukin-1 β processing, indicating that it is a dominant negative inhibitor of ICE itself.

In the present experiments we demonstrated that transgenic mice expressing the dominant negative ICE inhibitor are resistant to MPTP neurotoxicity. Following administration of MPTP there was a significant reduction of dopamine, DOPAC and HVA in the control mice, whereas this was completely inhibited in the ICE C285G mutant mice. There was also complete protection against a loss of tyrosine hydroxylase-immunoreactive neurons in the substantia nigra. Furthermore we demonstrated that this effect was not due to an aberration in MPTP uptake or processing since MPP⁺ levels were

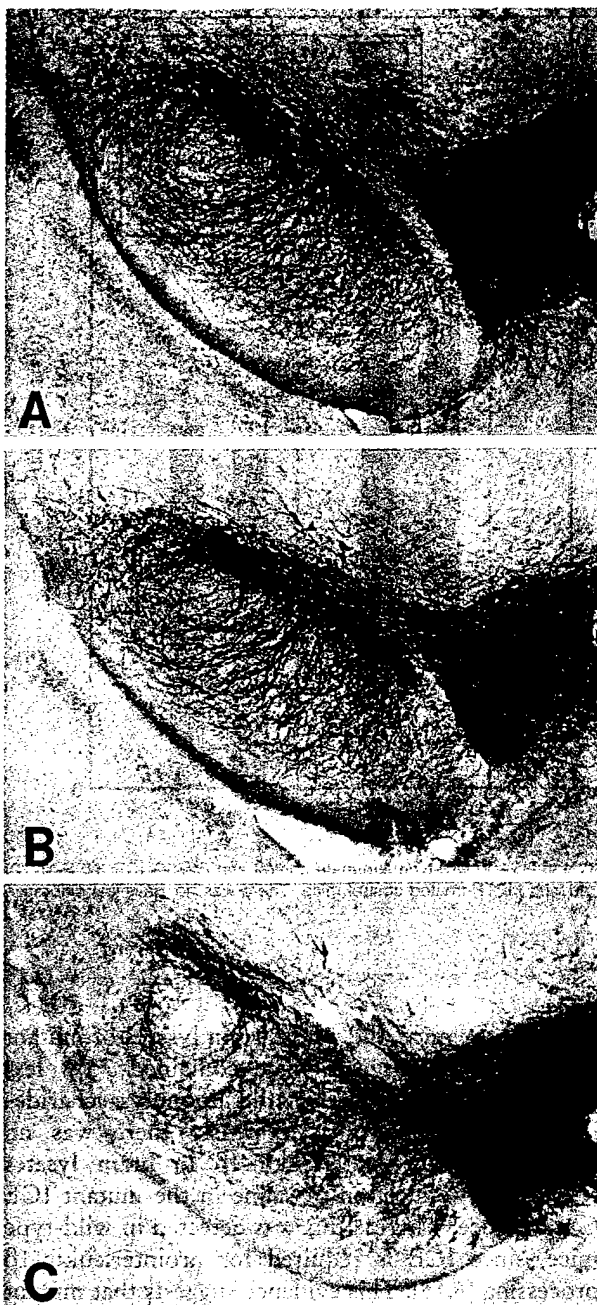


FIG. 2. Photomicrographs of tyrosine hydroxylase (TH) immunostaining of the substantia nigra from wild-type littermate control (A), C285G ICE/MPTP-treated (B) and wild-type littermate control/MPTP-treated (C) mice. There were no differences in immunostaining intensity and the numbers of TH-positive neurons were observed between the untreated control (A) and the C285G ICE/MPTP-treated mice (B). The wild-type littermate control/MPTP-treated mice (C) showed significant neuronal loss and diminished TH immunoreactivity particularly within the medial segment of the substantia nigra.

similar in both the littermate control and the ICE C285G mice.

The present results provide further evidence implicating ICE-mediated apoptotic cell death in MPTP neurotoxicity. We show that a mouse model which is deficient in ICE is resistant to MPTP neurotoxicity. This is consistent with other recent data which demonstrated that ICE C285G mice were resistant to permanent, as well as transient, middle cerebral artery occlusion and that infarct volume was significantly smaller [7,16]. It has also been demonstrated that crossing the ICE C285G mice with a transgenic mouse model of ALS significantly extended their survival [17].

Conclusion

The present results are consistent with a role of ICE-mediated apoptotic cell death in MPTP neurotoxicity. They provide further evidence for a role of apoptosis in MPTP-mediated toxicity, as reported previously [14]. These results suggest that similar mechanisms might be occurring in PD. If this is indeed the case then agents which could inhibit caspase-mediated apoptotic cell death might prove useful in the treatment of PD.

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Manganese Superoxide Dismutase Overexpression Attenuates MPTP Toxicity

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There is substantial evidence implicating mitochondrial dysfunction and free radical generation in the neurotoxicity of MPTP. Manganese superoxide dismutase (MnSOD) is the primary antioxidant enzyme protecting against superoxide radicals produced within mitochondria. Overexpression of human MnSOD in transgenic mice resulted in increased MnSOD localized to mitochondria in neurons and a 50% increase in total MnSOD activity in brain homogenates. We found that MPTP toxicity was significantly attenuated in the MnSOD transgenic mice which overexpress the human manganese superoxide dismutase gene, with these mice showing threefold greater dopamine levels than controls following MPTP. There were no alterations in MPP⁺ levels, suggesting that the effects were not due to altered metabolism of MPTP. A significant increase in 3-nitrotyrosine levels was seen in littermate controls but not in transgenic mice overexpressing human MnSOD. These results provide further evidence implicating mitochondrial dysfunction and oxidative damage in the pathogenesis of MPTP neurotoxicity.

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Key Words: MPTP; Parkinson's disease; free radicals; oxidative damage; superoxide dismutase.

Mitochondria consume over 90% of the cell's oxygen, and the mitochondrial respiratory chain is a major source of superoxide radicals (Boveris & Chance, 1973). Several different superoxide dismutases (SOD) have evolved to inactivate both intracellular and extracellular superoxide. The intracellular superoxide dismutases consist of MnSOD, which is localized within the mitochondrial matrix, and copper- and zinc-containing SOD, localized predominantly in cytoplasmic and nuclear compartments (Weisiger & Fridovich, 1973; Beyer *et al.*, 1991). Another copper- and zinc-containing superoxide dismutase was found predominantly in extracellular compartments (Marklund, 1982). MnSOD plays a critical role in normal antioxidant function since mice which are deficient develop neurodegeneration, myocardial injury, and perinatal death (Li *et al.*, 1995; Lebovitz *et al.*, 1996). Furthermore mice with a partial deficiency of MnSOD show increased

superoxide radical levels and neuronal degeneration after a permanent focal stroke (Murakami *et al.*, 1998).

There is substantial evidence that MPTP toxicity involves impairment of mitochondrial function (Tipton & Singer, 1993). MPTP is metabolized by monoamine oxidase B to 1-methyl-4-phenylpyridinium (MPP⁺). MPP⁺ once formed is taken up into dopaminergic neurons by the synaptic dopamine transporter. MPP⁺ then accumulates in mitochondria where it inhibits complex I of the electron transport chain. This may then lead to a pathologic cascade involving both excitotoxicity and free radical generation (Sriram *et al.*, 1997; Hasegawa *et al.*, 1990).

If the mitochondria are the primary source of superoxide radical generated by MPTP then one would expect that mice overexpressing the MnSOD would show an attenuation of neurotoxicity. In the present study we examined whether mice overexpressing the

human MnSOD gene show significant neuroprotection against MPTP-induced depletion of dopamine levels, as well as increases in 3-nitrotyrosine generation, a marker for peroxynitrite-mediated oxidative damage.

METHODS

The construction of the transgenic mice has been previously described (Yen *et al.*, 1996). A transgene containing the human MnSOD cDNA and the human β -actin 5' flanking sequence and promoter was constructed. The heterozygote transgenic mice and littermate controls were genotyped by Southern analysis. Transgenic mice were found to have increased expression of MnSOD transgene in numerous tissues. Immunogold staining showed that the human MnSOD is localized to mitochondria. Initial studies show that it resulted in a twofold increase in MnSOD activity in heart tissues of transgenic mice and this resulted in significant protection against adriamycin-induced cardiac toxicity (Yen *et al.*, 1996).

The presence of MnSOD in cortical neurons was examined using immunogold immunocytochemistry. Brain tissue was fixed for 1 h in Carson Millonig's fixative (4% formaldehyde on 0.16 M monobasic sodium phosphate buffer (pH 7.2)) and embedded in LR white acrylic resin according to the method of Mutasa (1989). Samples to be embedded were partially dehydrated in 70% ethanol before immersion and infiltration in undiluted LR white resin. All dehydration and infiltration steps with LR white were carried out at 25°C. Resin polymerization was thermal induced in sealed gelatin capsules at 40°C for 40 h in the absence of accelerator. Ultrathin sections (70–80 nm) were cut on a Sorvall MT2-B ultramicrotome with a Diatome diamond knife and subsequently transferred to 300 nm nickel grids for immunolabeling.

Nonspecific antibody binding was blocked by treating the sections with a 2% BSA solution (wt/vol) in TBS supplemented with 0.2% Tween 20 and 0.05% NaN_3 and then immunostained with the primary antiserum at a dilution range of 1:50–1:200 in 0.1% BSA in TBS for 18 h at 4°C in a humidified atmosphere. Sections were briefly washed and then reacted with a 1:50 dilution of gold-conjugated (10 nm) goat anti-rabbit IgG in 0.1% BSA in TBS (pH 8.2); the sections were fixed in 2.5% glutaraldehyde, washed extensively in double-distilled water, and counterstained with 4% aqueous uranyl acetate for 10 min. All electron micro-

scopic observations were made with a Philips 301 transmission electron microscope operated at 60 kV. To assess the specificity of immunolabeling, controls were performed by replacement of primary antiserum with preimmune rabbit serum.

An increase in MnSOD activity was determined using measurements in brain homogenates as well as on a slab gel. Brain tissues were homogenized in 4 vol of 0.05 M phosphate buffer, pH 7.8, in a Dounce homogenizer. Homogenization was followed by sonic disruption for 3 min in 15-s bursts using a microtip sonic dismembrator with cooling. Sodium cyanide (5 mM Na) inhibits CuZnSOD; therefore, activity measured in the crude homogenate in the presence of CN measures only MnSOD activity. The activity was assayed by the nitroblue tetrazolium (NBT) reduction method originally described by Beauchamp and Fridovich (1971) with the modifications described by Spitz and Oberley (1989). Briefly, aliquots of the homogenate were added to spectrophotometer cuvettes containing 1 mM diethylenetriamine pentaacetic acid, 5.6×10^5 M NBT, and 1 unit of catalase in 50 mM phosphate buffer, pH 7.8. The reaction was started with approximately 10^{-3} units of xanthine oxidase. The rate of increase in the absorbance at 560 nm indicates the rate at which the superoxide radical reduces NBT to blue formazan (BF). The amount of xanthine oxidase was adjusted so that the absorbance change was approximately 0.02/min. MnSOD inhibits the superoxide-dependent reduction of NBT. One unit is defined as the amount of MnSOD which causes a 50% inhibition of the reduction of NBT to BF. The MnSOD-specific activity is reported as units/mg protein.

The second method for measuring SOD activity involves running nondissociating, vertical, slab gels by the method of Beauchamp and Fridovich (1971) with slight modifications. Ammonium persulfate was used as the initiator in the running gel (7.5% polyacrylamide), and riboflavin-light was used for the stacking gel (3.5% polyacrylamide). After electrophoresis, MnSOD activity was localized by soaking the gels in 2.45×10^{-3} M NBT containing 5 mM CN for 20 min and then immersing for 15 min in a solution containing 0.028 M tetramethylethylenediamine, 2.8×10^{-5} M riboflavin, and 0.036 M K_2PO_4 at pH 7.8. After decanting the solution, the gels were wrapped in Saran Wrap, illuminated with fluorescent light, and photographed.

For MPTP toxicity we utilized mice overexpressing MnSOD as well as littermate controls. Ten mice in each group were injected with phosphate-buffered saline

and served as baseline controls, and 10 mice in each group were injected with MPTP at a dose of 15 mg/kg every 2 h for five injections. MPTP (Research Biochemicals, Wayland, MA) was administered in 0.1 ml of water. MPTP solution was prepared in a hood using masks and gloves. Mice were sacrificed at 1 week and the striata were dissected and sonicated in 0.1 M perchloric acid and centrifuged, and dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were analyzed by HPLC with electrochemical detection (Beal *et al.*, 1990).

In a follow-up experiment 10 animals in each group were administered two doses of MPTP of 30 mg/kg i.p. 2 h apart and sacrificed at 2 h after the last dose. Both striata were dissected for MPP⁺ and 3-nitrotyrosine measurements. MPP⁺ levels were quantified by HPLC with uv detection at 295 nm. Samples were sonicated in 0.1 M perchloric acid and an aliquot of supernatant was injected onto a Brownlee aquapore XO3-224 cation-exchange column (Rainin, Woburn, MA). Samples were eluted isocratically with 90% 0.1 M acetic acid, 75 mM triethylamine HCl (pH 2.35, adjusted with formic acid), and 10% acetonitrile. The flow rate was 1 ml/min. 3-Nitrotyrosine and tyrosine concentrations were quantified by HPLC with 16-electrode electrochemical detection as previously described (Schulz *et al.*, 1995). The results are expressed as the mean \pm standard error of the mean. Statistical comparisons were made by Student's *t* test or one-way analysis of variance followed by Fischer PLSD test.

RESULTS

The results of immunogold electron microscopy for localization of MnSOD in the transgenic mice and wild-type controls are shown in Fig. 1. MnSOD was confined to mitochondria in both the wild-type (Fig. 1a) and the transgenic (Fig. 1c) mice. There was an increase in immunogold labeling in the transgenic mice compared to wild-type controls. Nonimmune serum resulted in no labeling in either wild-type or transgenic mice (Figs. 1b and 1d). As shown in Fig. 2, a slab gel assay showed increased activity for human MnSOD in the transgenic mice. The total manganese SOD activity (mouse and human) in brain homogenates of the wild-type mice was 20.8 ± 0.8 units/mg protein and in the transgenics it was 31.6 ± 4.6 units/mg protein ($P < 0.016$).

As shown in Fig. 3, mice which overexpress human

manganese superoxide dismutase showed significant attenuation of MPTP-induced dopamine depletions and DOPAC depletions. A similar trend was seen with homovanillic acid concentrations; however, this did not reach statistical significance. The MPP⁺ levels in controls were 20.3 ± 3.7 ng/mg protein, and in the MnSOD-overexpressing mice they were 24.8 ± 4.0 ng/mg protein, $P = 0.42$. As shown in Fig. 4, 3-nitrotyrosine concentrations were significantly increased at 2 h after MPTP administration in the littermate control mice. In the mice overexpressing human MnSOD there was a small increase in 3-nitrotyrosine which did not reach statistical significance.

DISCUSSION

There is substantial evidence that MPTP neurotoxicity involves an impairment of energy metabolism followed by increased free radical generation. Previous studies have demonstrated increased free radical generation associated with MPP⁺ *in vitro* (Hasegawa *et al.*, 1990) and with MPTP *in vivo* (Sriram *et al.*, 1997; Smith & Bennett, 1997). Furthermore there is evidence that antioxidants can attenuate MPTP-induced neurotoxicity. It was shown that mice which have a two- to threefold increase in copper/zinc superoxide dismutase activity in cerebral cortex show almost complete protection against MPTP toxicity (Przedborski *et al.*, 1992).

If the site of increased free radical production following MPTP administration is the mitochondria, then one might expect that mice that overexpress MnSOD might show protection against MPTP-induced dopamine depletions. We utilized transgenic mice which overexpress human MnSOD. Immunogold electron microscopy confirmed that there was increased labeling of MnSOD confined to mitochondria in cortical neurons of these mice. A gel total activity assay verified the presence of human MnSOD in transgenic mouse brain. Enzymatic activity measurements in brain homogenates confirmed an increase in MnSOD activity of about 50%. These mice show reduced lipid peroxidation, protein nitration, and neuronal death after focal cerebral ischemia (Keller *et al.*, 1998). Compared with littermate controls, mice that overexpressed human MnSOD showed significant protection against MPTP-induced depletions of dopamine and DOPAC. Protection against depletion of HVA did not quite reach significance. The extent of the protection

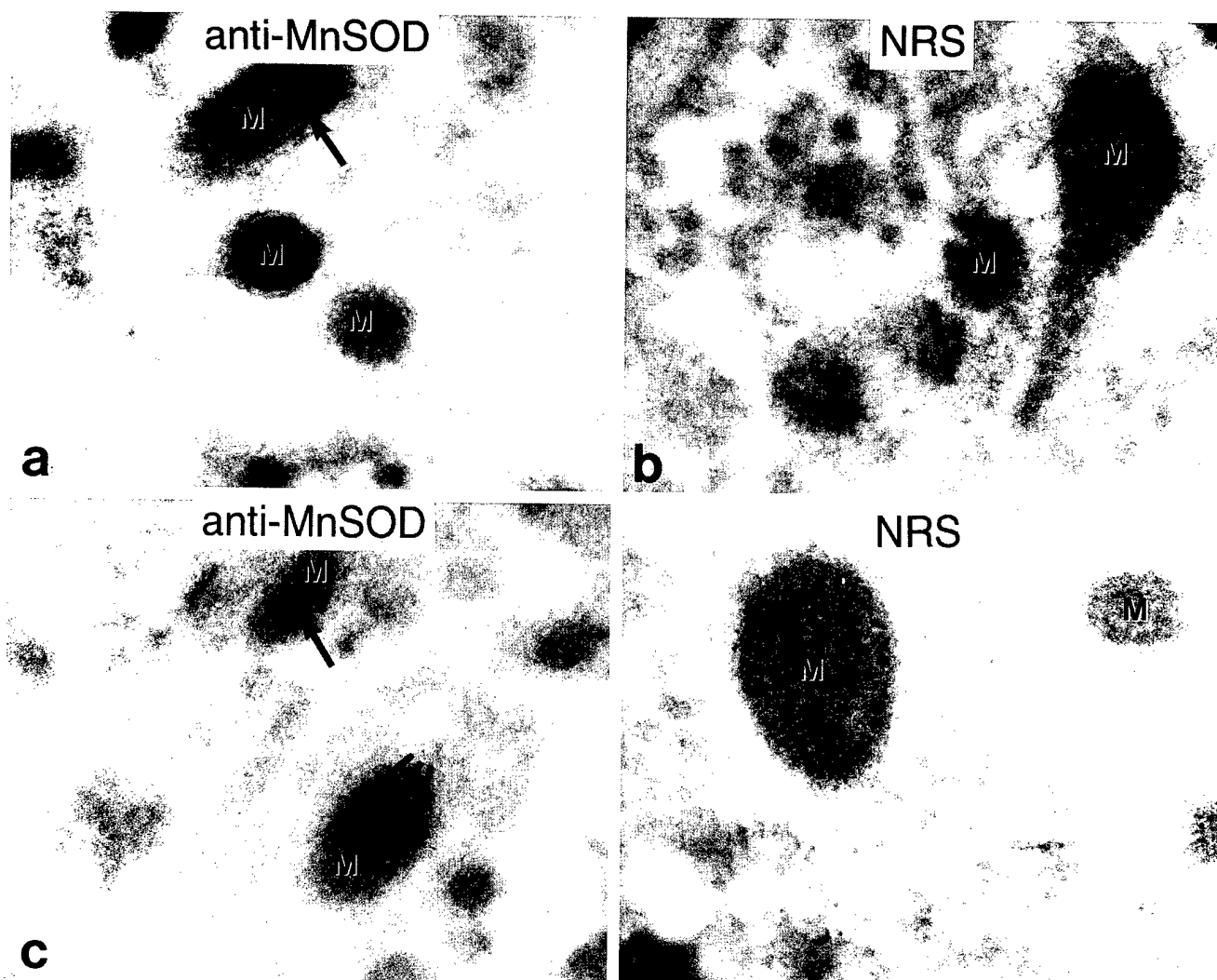


FIG. 1. Immunogold electron microscopy of MnSOD in mouse cortical neurons. (a) A wild-type mouse showing scattered gold beads over mitochondria (M), but no staining in cytoplasm; (b) a wild-type mouse labeled with nonimmune serum showing no labeling of mitochondria; (c) a MnSOD transgenic mouse showing numerous gold beads over mitochondria (M), but no staining of cytoplasm, and (d) a MnSOD transgenic mouse showing no staining of mitochondria with nonimmune serum.

was not as marked as that seen in mice which overexpress copper/zinc superoxide dismutase. This, however, may be due to the degree of expression of the human MnSOD in cerebral cortex of the transgenic mice, which is not as great an increase as that seen in the mice overexpressing copper/zinc superoxide dismutase studied previously (Przedborski *et al.*, 1992). The neuroprotective effects do not appear to be due to altered MPTP uptake or metabolism since MPP⁺ levels were comparable at 2 h in the MnSOD-overexpressing mice compared to littermate controls.

We examined whether the mechanism of the protection is associated with a reduction in oxidative dam-

age. We previously showed that MPTP administration is associated with increased 3-nitrotyrosine concentrations in the striatum (Schulz *et al.*, 1995). 3-Nitrotyrosine is thought to be a relatively specific marker of peroxynitrite-mediated nitration (Ischiropoulos *et al.*, 1992). Peroxynitrite is produced by the reaction of nitric oxide (NO) with superoxide (O₂⁻) (Beckman & Crow, 1993). We and others found previously that neuronal nitric oxide synthase inhibitors which block the generation of peroxynitrite protect against MPTP toxicity both in mice and in primates (Schulz *et al.*, 1995; Hantraye *et al.*, 1996; Przedborski *et al.*, 1996). In the present study we found significant MPTP-induced

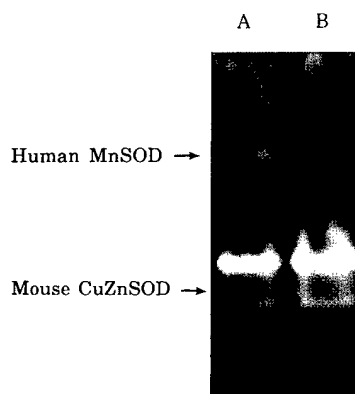


FIG. 2. A native polyacrylamide gel stained for brain SOD activity in a MnSOD transgenic (A) and a wild-type mouse (B). The middle and lower bands are both mouse CuZnSOD.

increases in 3-nitrotyrosine in the littermate control mice; however, mice which overexpress MnSOD showed no significant increase in concentrations. These findings therefore provide direct evidence that overexpression of MnSOD in brain can protect against peroxynitrite-mediated oxidative damage.

There is substantial evidence that oxidative damage may play a role in idiopathic Parkinson's disease (PD) (Beal, 1997). There is evidence for increased lipid peroxidation, protein oxidation, and oxidative damage to DNA in Parkinson's disease substantia nigra. There is also evidence for a deficiency of complex I of the electron transport chain in PD substantia nigra. As noted above, previous studies have demonstrated that oxidative damage also appears to play a role in MPTP neurotoxicity. The present results provide further evidence implicating oxidative damage produced at the

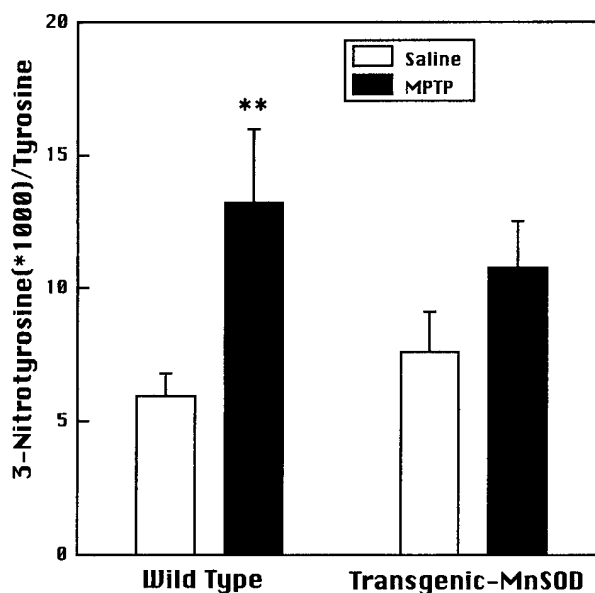


FIG. 4. Effects of MPTP administration on 3-nitrotyrosine levels in littermate controls and transgenic mice overexpressing manganese superoxide dismutase. ** $P < 0.01$ compared with saline-treated controls.

level of the mitochondria in the pathogenesis of MPTP neurotoxicity and as such suggest that similar mechanisms may occur in Parkinson's disease.

ACKNOWLEDGMENTS

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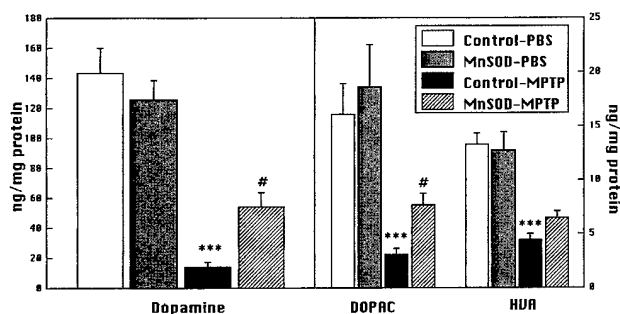


FIG. 3. Effects of MPTP (15 mg/kg \times 5 doses) on dopamine, DOPAC, and HVA in littermate controls and transgenic mice overexpressing manganese superoxide dismutase. *** $P < 0.001$ compared with littermate controls. # $P < 0.05$ compared to MPTP-treated littermate controls.

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Creatine and Cyclocreatine Attenuate MPTP Neurotoxicity

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Systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) produces parkinsonism in experimental animals by a mechanism involving impaired energy production. MPTP is converted by monoamine oxidase B to 1-methyl-4-phenylpyridinium (MPP⁺), which blocks complex I of the electron transport chain. Oral supplementation with creatine or cyclocreatine, which are substrates for creatine kinase, may increase phosphocreatine (PCr) or cyclophosphocreatine (PCCr) and buffer against ATP depletion and thereby exert neuroprotective effects. In the present study we found that oral supplementation with either creatine or cyclocreatine produced significant protection against MPTP-induced dopamine depletions in mice. Creatine protected against MPTP-induced loss of Nissl and tyrosine hydroxylase immunostained neurons in the substantia nigra. Creatine and cyclocreatine had no effects on the conversion of MPTP to MPP⁺ *in vivo*. These results further implicate metabolic dysfunction in MPTP neurotoxicity and suggest a novel therapeutic approach, which may have applicability for Parkinson's disease. © 1999 Academic Press

Key Words: Parkinson's; creatine; cyclocreatine; MPTP; free radicals; mitochondria; phosphocreatine.

INTRODUCTION

MPTP is a neurotoxin that produces a parkinsonian syndrome in both man and experimental animals. Studies of the mechanism of MPTP neurotoxicity showed that it is not the true neurotoxic agent. It was demonstrated that 1-methyl-4-phenylpyridinium (MPP⁺) is produced by the metabolism of MPTP by monoamine oxidase B (34). Inhibitors of monoamine oxidase B block the neurotoxicity of MPTP in both mice and primates (17, 21).

MPP⁺ once formed is taken up into dopaminergic neurons by the synaptic dopamine transporter (14). MPP⁺ then accumulates in mitochondria where it inhibits complex I of the electron transport chain (15). *In vitro* studies showed that it produces an impairment

of oxidative phosphorylation and structure-activity studies with MPP⁺ analogs showed that their ability to inhibit respiration in cultured dopaminergic neurons correlates well with their neurotoxic potential (15). *In vivo* studies showed that systemic administration of MPTP depletes striatal concentrations of ATP, as do intrastriatal injections of MPP⁺ (8, 32). This may lead to a pathologic cascade involving both excitotoxicity and free radical generation (3, 6, 29–31).

If the pathologic cascade involves an initial impairment of energy metabolism then strategies to either improve oxidative phosphorylation, or to buffer ATP depletion, might prove effective in attenuating MPTP neurotoxicity. The major energy store in the brain is ATP, which is tightly coupled to the creatine kinase system. Creatine kinase (CK) is a key enzyme involved in regulating energy metabolism in cells with intermittently high and fluctuating energy requirements including the brain. The enzyme catalyzes the reversible transfer of the phosphoryl group from phosphocreatine (PCr) to ADP, to generate ATP (36). Several cytoplasmic and mitochondrial isoforms have been identified and along with the substrates Cr and PCr constitute an intricate cellular energy buffering and transport system connecting sites of energy production with sites of energy consumption (18).

The mitochondrial isoform creatine kinase (Mi-CK) is located at contact sites between the inner and outer membranes where it is associated with porin (5, 36). Mi-CK can directly convert intramitochondrially produced ATP to PCr, which then gets transported to sites of energy consumption. There is much evidence to suggest that the CK system is important in regulating energy homeostasis in the brain. Creatine has been shown to effectively stimulate mitochondrial state 3 respiration leading to net production of PCr (20, 25). Both creatine and its analogue cyclocreatine modulate rates of ATP production through the CK system. We therefore examined whether oral administration of either creatine or cyclocreatine could exert neuroprotective effects against MPTP-induced dopamine deple-

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tions and whether they reduce MPTP-induced free radical generation.

METHODS

To determine the optimal doses of creatine and cyclocreatine that protect against MPTP toxicity, a pilot experiment was performed in which male Swiss Webster mice were orally administered creatine and cyclocreatine at 0.25–3.0% in the diet for 2 weeks prior to MPTP administration. Controls received otherwise identical diets without creatine and cyclocreatine. MPTP was injected i.p. at a dose of 15 mg/kg every 2 h for 5 injections. From this initial study, it was determined that 1% creatine and 1% cyclocreatine provided optimal protection against MPTP injections (see Fig. 1). In a second experiment, three groups of mice, each composed of 12 animals, were fed lab chow diets supplemented with 1% creatine, 1% cyclocreatine, or a standard unsupplemented diet for 2 weeks prior to MPTP administration (see Fig. 2). To ensure MPTP toxicity, animals were injected with 15 mg/kg every 2 h for 6 injections. MPTP (Research Biochemicals, Wayland, MA) was administered in 0.1 ml of water. Animals remained on their respective supplemented or unsupplemented diets for 1 week after MPTP injections at which time they were sacrificed. Following sacrifice the two striata were rapidly dissected and placed in chilled 0.1 M perchloric acid and taken for protein quantification using a fluorimetric assay, while the substantia nigras were postfixed in buffered 4% paraformaldehyde and used for immunocytochemical and routine histological staining. Dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were quantified by HPLC with 16 electrode electrochemical detection (4).

In order to determine whether or not creatine or cyclocreatine altered MPTP levels, eight animals in each group were fed with either a normal diet, 1% creatine, or 1% cyclocreatine for 2 weeks prior to injection of MPTP at 30 mg/kg i.p. for analysis of MPP⁺ levels. The animals were sacrificed at 90 min and both striata were dissected. MPP⁺ levels were quantified by HPLC with UV detection at 295 nm. Samples were sonicated in 0.1 M perchloric acid and an aliquot of supernatant was injected onto a Brownlee aquapore X03-224 cation exchange column (Rainin). Samples were eluted isocratically with 90% 0.1 M acetic acid 75 mM triethylamine HCl (pH 2.35, adjusted with formic acid) and 10% acetonitrile. The flow rate was 1 ml/min.

We examined the effects of creatine feeding on brain concentrations of creatine in a separate experiment. Ten animals in each group were fed with normal diets, 0.5, 1, or 2% creatine for 3 weeks. They were sacrificed by a freeze-clamp procedure and creatine levels were measured as previously described (23).

In the second experiment the midbrain blocks containing the substantia nigra were postfixed in 4% buffered paraformaldehyde. Ten littermate control, creatine-MPTP, and MPTP animals were examined. One week after MPTP injections all animals were deeply anesthetized with pentobarbital and transcardially perfused with cold (4°C) saline, followed by cold 0.1 M phosphate-buffered 4% paraformaldehyde/lysine/sodium *m*-periodate solution for histopathologic evaluation. The whole brain specimens were removed and cryoprotected in 15 and 20% glycerol solution, respectively, made in 0.1 M phosphate buffer and 2% DMSO. Each brain was serially sectioned coronally at 50 μ m and collected into six well chambers. All sections from individual well chambers were subsequently stained for routine cell identification using cresyl violet (Nissl) and immunocytochemically for tyrosine hydroxylase (TH) (TH antisera; 1:1,000 dilution; Eugene Tech International, Inc.). The immunohistologic methods used in this study have previously been reported (12).

Immunocytochemistry

Immunocytochemistry was performed on 50- μ m-thick tissue sections, using a conjugated second antibody method. The procedure was as follows: the tissue sections were preincubated in absolute methanol 0.3% hydrogen peroxide solution for 30 min, washed (3 \times) in phosphate-buffered saline (PBS) (pH 7.4) 10 min each, placed in 10% normal goat serum (Gibco Labs) for 1 h, incubated free floating in primary antiserum at room temperature for 12–18 h (all dilutions of primary antisera above included 0.3% Triton X-100 and 10% normal goat serum), washed (3 \times) in PBS for 10 min each, placed in periodate-conjugated goat anti-rabbit IgG (1:300 in PBS) (Boehringer-Mannheim), washed (3 \times) in PBS 10 min each, and reacted with 3,3'-diaminobenzidine HCl (1 mg/ml) in Tris-HCl buffer with 0.005% hydrogen peroxide. The TH antibody has previously been well characterized and tested by preadsorption of dilute primary antisera with an excess of appropriate antigen for 6 h at room temperature prior to incubation or by elimination from the primary incubation solution.

Midbrain sections (two or three microscopic sections) through both the left and right substantia nigras (Bregma levels –2.92 mm to 3.08 mm; intraaural levels 0.88 mm to 0.72 mm) (13) in each of the MPTP-treated, creatine/MPTP-treated, and littermate control mice were scanned by microscopic videocapture and analyzed. Stereological analysis of Nissl stained neurons and TH-positive neurons within the substantia nigra pars compacta was completed using Neurolucida software (Microbrightfield). Cell counts were made and corrected for tissue section thickness and volume changes. Statistical analysis was made by using ANOVA followed by the Fisher protected least significant differences post-hoc test. The results are expressed as

means \pm SEM. All animals used in these procedures were in strict compliance with the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by all local Animal Care Committees.

RESULTS

Initial pilot experiments showed that both 1% creatine and 1% cyclocreatine produced significant protection against MPTP-induced dopamine depletion. We carried out further experiments with 0.25–3% creatine supplementation in the diet (Fig. 1). MPTP was administered at 15 mg/kg i.p. for five doses. Doses of 0.25, 0.5, and 1% creatine exerted dose-dependent significant neuroprotective effects, which disappeared at doses of 2 and 3% creatine, consistent with a U-shaped dose-response curve. Cyclocreatine exerted significant protection against dopamine depletions at doses of 0.5 and 1% cyclocreatine in the diet. Effects of creatine on the dopamine metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) paralleled those seen with dopamine. Cyclocreatine also exerted neuroprotective effects against HVA and DOPAC, although protection against HVA depletion was not seen with 0.5% cyclocreatine, presumably due to experimental variability. In a follow-up experiment we examined the effects of 1% creatine and 1% cyclocreatine on MPTP at 15 mg/kg i.p. for six doses. With this dosing regimen there was a 70% depletion of dopamine as

compared with controls (Fig. 2). Administration of both creatine and cyclocreatine produced marked significant protection against MPTP-induced depletions of dopamine, DOPAC, and HVA (F values = 22.6, 6.8, and 7.6, respectively).

Feeding with either 1% creatine or 1% cyclocreatine had no significant effect on MPP⁺ levels at 90 min after administration of MPTP. The MPP⁺ levels in controls were 36.0 ± 4.5 ng/mg protein, with 1% creatine they were 31.5 ± 3.5 ng/mg protein and with 1% cyclocreatine they were 40.5 ± 8.5 ng/mg protein. We found that feeding with creatine for 3 weeks dose-dependently significantly increased brain creatine levels ($P < 0.001$, $F = 5.34$). The levels were 139.1 ± 6.2 , 131.8 ± 3.9 , 147.6 ± 7.4 , and 174.3 ± 10.2 μ mol/g protein, respectively, in mice on control, 0.5, 1, and 2% creatine supplemented diets.

Histologic examination of serially cut, Nissl-stained, and TH-positive immunostained sections through the midbrains of control, creatine/MPTP-treated, and MPTP-treated mice showed that in comparison to control and creatine/MPTP-treated mice, there were selective bilateral lesions within the substantia nigra in the MPTP-treated group of animals (Figs. 3 and 4). There was no significant loss of nigral neurons in creatine/MPTP-treated animals, while the severity of neuronal loss within the substantia nigra pars compacta in MPTP-treated animals was marked, especially within the medial aspect (Figs. 3 and 4). Neuronal

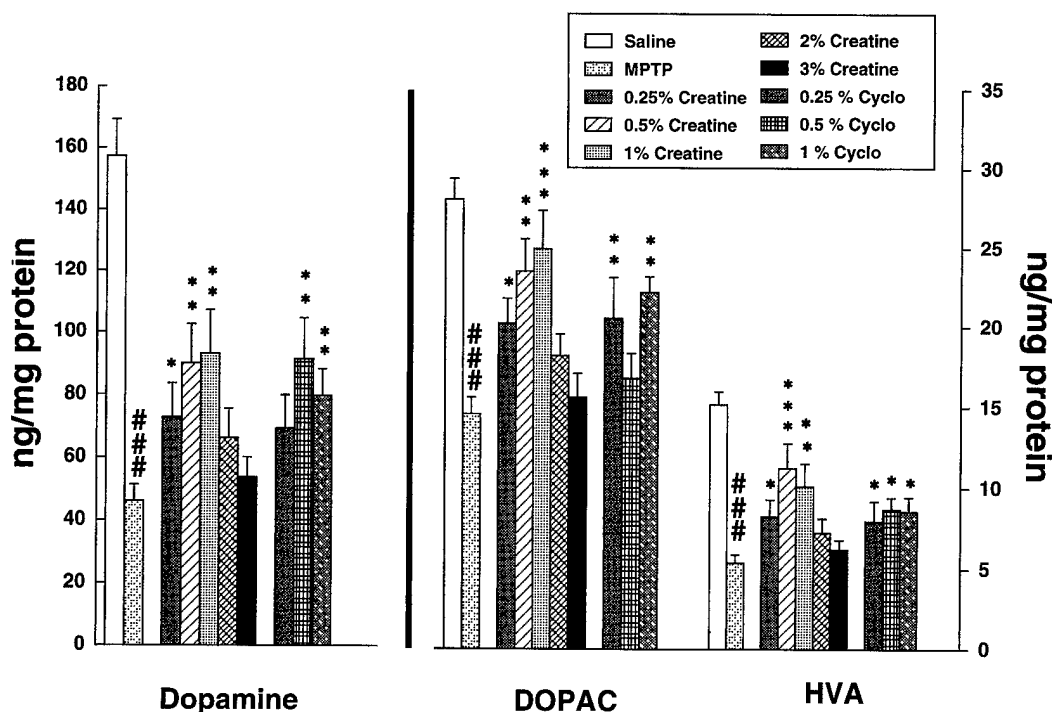


FIG. 1. Effects of increasing doses of creatine and cyclocreatine on MPTP (15 mg/kg \times 5 doses) induced depletions of dopamine, DOPAC, and HVA. ### $P < 0.001$ compared with control, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with MPTP, $n = 10$ per group.

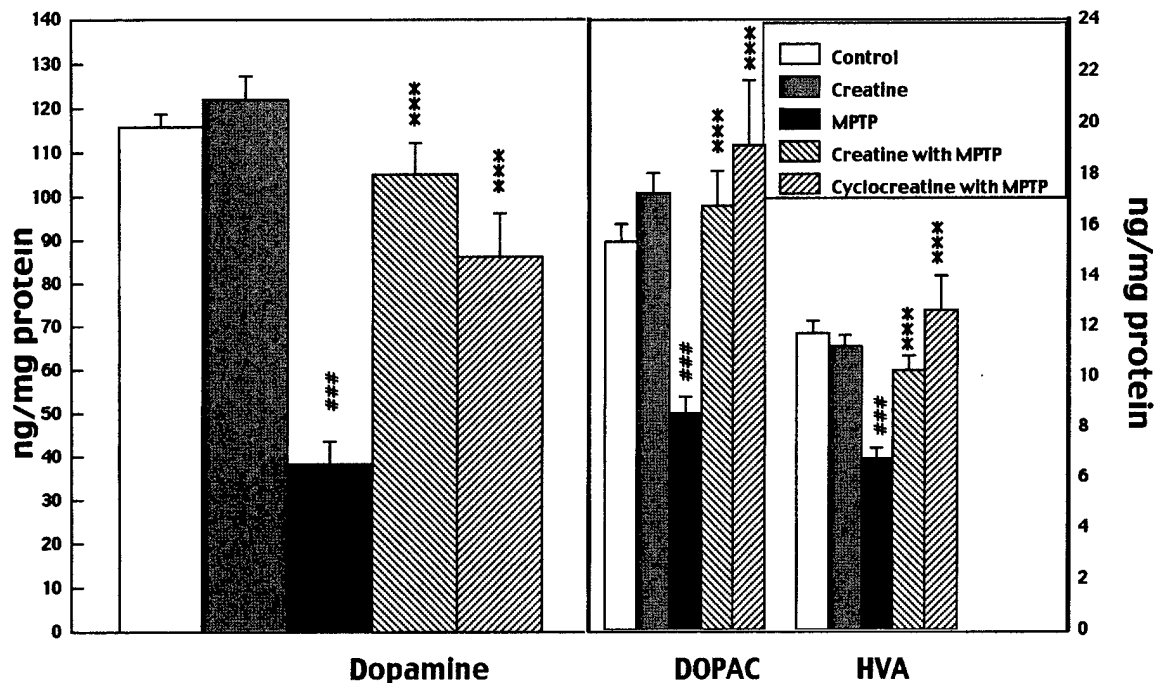


FIG. 2. Effects of 1% creatine or 1% cyclocreatine on MPTP (15 mg/kg \times 6 doses) induced depletions of dopamine, DOPAC, and HVA. ### P < 0.001 as compared with control, *** P < 0.001 as compared with MPTP, n = 12 per group.

counts of both Nissl- and TH-stained sections within the substantia nigra pars compacta revealed no significant differences between the control and creatine/MPTP-treated mice. A statistically significant difference (P < 0.01) between controls and creatine/MPTP animals as compared with the MPTP-treated mice was observed (Table 1).

DISCUSSION

The major energy source in the brain is ATP, which is tightly coupled to PCr. CK catalyzes the reaction of ADP with PCr to generate ATP. The brain isoform of CK along with the mitochondrial isoform and the substances creatine and PCr constitute a system that seems to be critical in regulating energy homeostasis in the brain and other organs with high and fluctuating energy demands (36). This is consistent with the finding that high energy turnover and high CK concentrations have been found in those regions of the brain that are rich in synaptic connections, e.g., molecular layer of the cerebellum, glomerular structures of the granule layer, and the hippocampus (19). ATP generated by oxidative phosphorylation is transported through the inner membrane of the mitochondria by the adenine nucleotide transporter, where it is transphosphorylated by the MiCK to generate PCr. PCr then leaves the mitochondrion and diffuses to the cytoplasm where it serves as both a temporal and spatial energy buffer (36). Creatine is an excellent stimulant for mitochon-

drial respiration resulting in the generation of PCr (20, 25). PCr maintains ATP levels utilized by the Na^+/K^+ ATPase and the Ca^{2+} ATPase (10, 18). The importance of CK function in the adult brain is supported by *in vivo* ^{31}P -NMR transfer measurements showing that the pseudo first-order rate constant of the CK reaction (in the direction of ATP synthesis), as well as the CK flux correlates with brain activity measured by EEG and 2-deoxyglucose uptake (9, 28). Both the brain cytosolic creatine kinase and the brain mitochondrial creatine kinase are widely distributed in all areas of the central nervous system.

There is substantial evidence that MPTP neurotoxicity involves an impairment of energy metabolism and ATP depletion (8, 32). If this is of pathogenic significance then one would expect that creatine or cyclocreatine administration might exert neuroprotective effects. Creatine and cyclocreatine stimulate the rate of ATP synthesis and produce high amounts of PCr or phosphocyclocreatine (PCCr). The build up of PCr (or PCCr) could help neurons sustain ATP levels for an extended period of time, especially under energy depletion or stress conditions. In heart and skeletal muscles cyclocreatine administration increased tissue levels of cyclocreatine and delayed both ischemia-induced depletion of ATP levels and tissue rigor (2, 11, 16, 26, 27, 35). In hippocampal slices creatine supplementation increased PCr, delayed synaptic failure, and attenuated anoxic damage (7, 37). Cyclocreatine administration appears to buffer depletion of ATP stores induced by

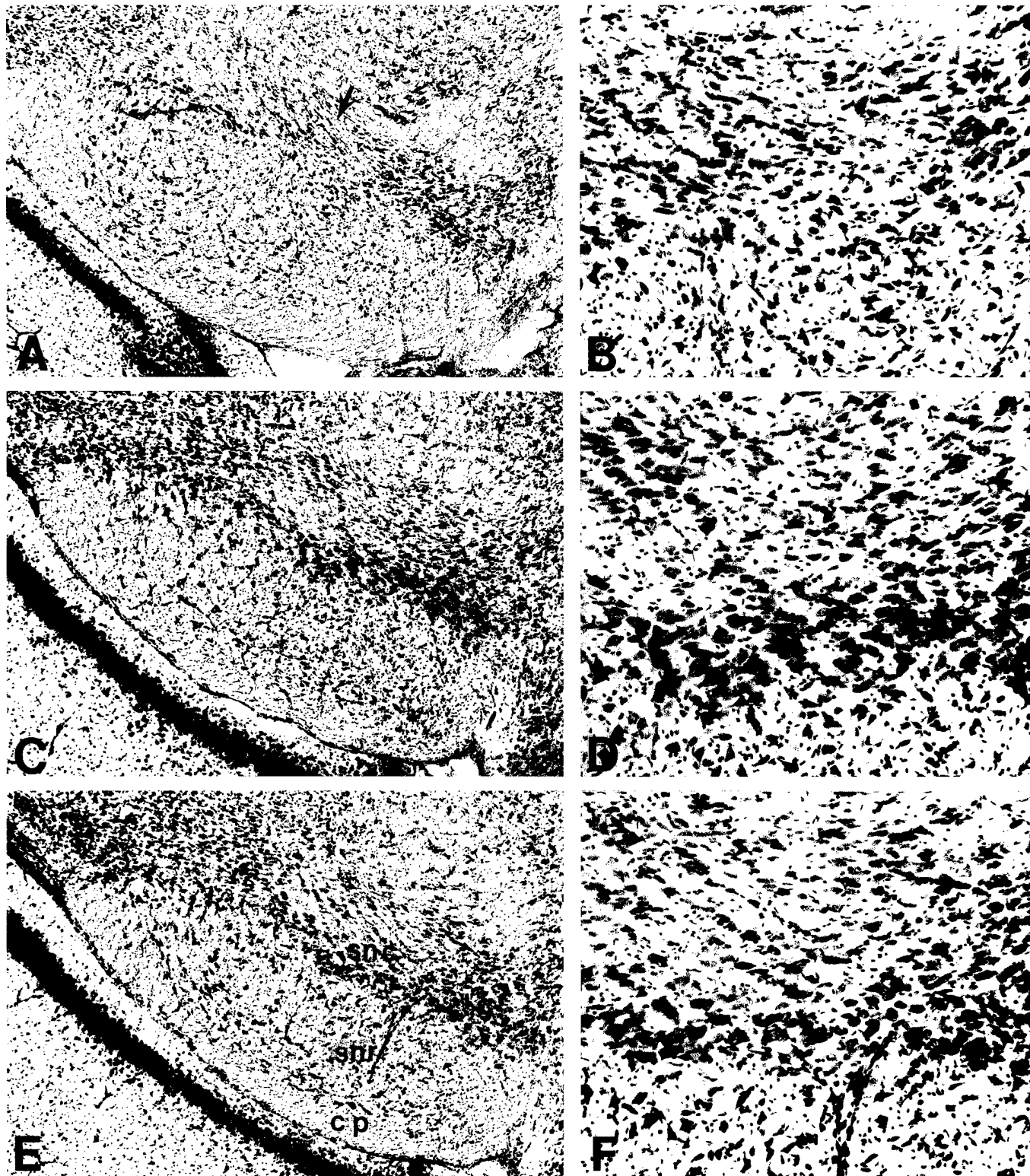


FIG. 3. Photomicrographs of the midbrain from MPTP-treated Nissl-stained sections of the substantia nigra from mice treated with MPTP alone show marked neuronal loss and gliosis within the medial segment of the substantia nigra pars compacta (A and B) (arrow) in comparison to animals prefed creatine and subsequently treated with MPTP (C and D) and a littermate control specimen (E and F). SNC, substantia nigra pars compacta; SNR, substantia nigra pars reticulata; cp, cerebral peduncle.

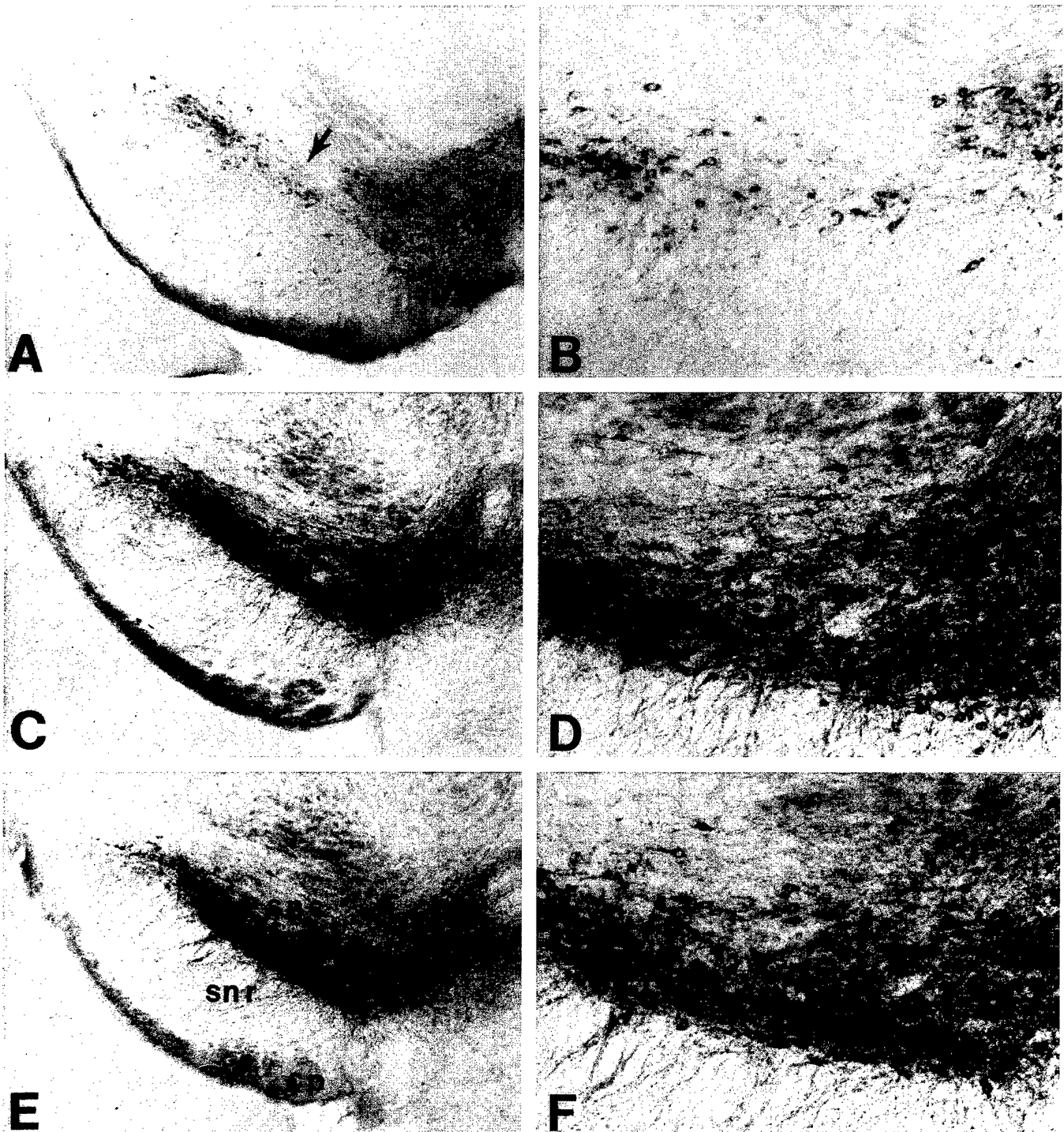


FIG. 4. Contiguous immunostained tissue sections from Fig. 3 for tyrosine hydroxylase (TH) immunocytochemistry show a significant loss of TH-positive neurons and their arbors in mice treated with MPTP alone (A and B) (arrow) within the substantia nigra compacta (SNC). In contrast, little or no loss of TH-positive neurons and immunoreactivity was observed within the substantia nigra in creatine fed animals (C and D) as compared to a littermate control specimen (E and F). SNC, substantia nigra pars compacta; SNR, substantia nigra pars reticulata; cp, cerebral peduncle.

TABLE 1

Nissl and Tyrosine Hydroxylase-Positive Neuronal Counts within the Substantia Nigra of MPTP, Creatine-MPTP, and Littermate Control Mice

	Nissl	TH
Control	142 ± 9.2	123 ± 4.1
Creatine-MPTP	135 ± 12.4	118 ± 6.3
MPTP	62 ± 18.8*	38 ± 10.6*

Note. In each of 10 controls, 10 creatine/MPTP-treated, and 10 MPTP-treated mice, stereologic counts of Nissl and tyrosine hydroxylase-positive neurons were made within the entire area of the substantia nigra pars compacta as identified. While no statistical significance was obtained between control and creatine/MPTP-treated specimens, there were significant reductions in both Nissl- and tyrosine hydroxylase-positive neurons in MPTP-treated animals as compared with both controls and creatine-MPTP-treated animals.

* $P < 0.01$.

ischemia (39). Creatine and cyclocreatine may also exert neuroprotective effects by enhancing glutamate uptake into synaptic vesicles and by stabilizing the mitochondrial transition pore (24, 40).

We found that both creatine and cyclocreatine produced dose-dependent neuroprotection against MPTP-induced dopamine depletion. Neuroprotective effects were also seen with the dopamine metabolites DOPAC and HVA. Creatine supplementation showed protection at 0.25, 0.5, and 1% in the diet, but none at 2 or 3%, suggesting an inverted dose-response curve. Cyclocreatine produced protective effects with doses of both 0.5 and 1.0%, with slightly better protection with 0.5%. The explanation for the U-shaped dose-response curve is unclear. We have observed a similar U-shaped curve with malonate toxicity (23). It is not due to an alteration in food intake and is not seen in all experimental paradigms. In a subsequent study using a different dosing regimen of MPTP 1% creatine or 1% cyclocreatine produced almost complete neuroprotection against depletion of dopamine and its metabolites. Both 1% creatine and 1% cyclocreatine also produced significant protection against MPTP-induced depletion of both Nissl- and tyrosine hydroxylase-stained neurons in the substantia nigra. The neuroprotective effects do not appear to be due to an effect of MPTP uptake or metabolism since MPP⁺ levels were comparable at both 3 and 6 h in animals fed with creatine.

Several studies have suggested that PD is associated with both bioenergetic defects and oxidative damage. Oxidative damage is widespread and could be a consequence of either a mitochondrial defect or of dopamine replacement therapy (1). A decrease in complex I activity of the electron transport chain has been reported in platelets, muscle, and the substantia nigra of PD patients (reviewed in Beal, 1995) (3). These findings suggest that a systemic defect in complex I activity may

play a role in the pathogenesis of PD. Consistent with this possibility the complex I defect in platelet mitochondria of PD patients can be transferred into mitochondria deficient cell lines, suggesting that it is a consequence of a mitochondrial DNA mutation (33). A recent study provided evidence for maternal inheritance in PD (38); however, others did not find evidence for maternal inheritance (22).

Several lines of evidence therefore implicate defective energy metabolism in the pathogenesis of both MPTP neurotoxicity and PD itself. Strategies to improve mitochondrial function might therefore be useful in the treatment of PD. We found that coenzyme Q₁₀, which may also enhance mitochondrial function, protects against MPTP toxicity (29). The present studies show that administration of either creatine or cyclocreatine can also attenuate MPTP neurotoxicity. If both MPTP neurotoxicity and PD involve similar pathogenic mechanisms, creatine or cyclocreatine administration may be a novel therapeutic strategy in attempting to slow the neurodegenerative process in PD patients.

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Novel Free Radical Spin Traps Protect against Malonate and MPTP Neurotoxicity

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Both malonate and 1-methyl-4-phenyl-1,2,5,6 tetrahydropyridine (MPTP) are neurotoxins which cause energy depletion, secondary excitotoxicity, and free radical generation. Malonate is a reversible inhibitor of succinate dehydrogenase, while MPTP is metabolized to 1-methyl-4-phenylpyridinium, an inhibitor of mitochondrial complex I. We examined the effects of pretreatment with the cyclic nitron free radical spin trap MDL 101,002 on malonate and MPTP neurotoxicity. MDL 101,002 produced dose-dependent neuroprotection against malonate-induced striatal lesions. MDL 101,002 produced significant protection against MPTP induced depletions of dopamine and its metabolites. MDL 101,002 also significantly attenuated MPTP-induced increases in striatal 3-nitrotyrosine concentrations. The free radical spin trap tempol also produced significant protection against MPTP neurotoxicity. These findings provide further evidence that free radical spin traps produce neuroprotective effects *in vivo* and suggest that they may be useful in the treatment of neurodegenerative diseases. © 1999 Academic Press

Key Words: free radicals; mitochondria; spin traps; Huntington's disease; Parkinson's disease.

INTRODUCTION

There is substantial evidence implicating a role of free radicals and oxidative stress in neurodegenerative diseases (Beal, 1997). Evidence comes from studies of postmortem tissue as well as from the study of animal models. We and others showed that intra-striatal administration of the reversible succinate dehydrogenase inhibitor malonate results in striatal lesions which mimic the neuropathological features of Huntington's disease (Henshaw *et al.*, 1994; Greene *et al.*, 1993; Beal, 1993). The lesions are accompanied by ATP depletion and they are blocked by excitatory amino acid antagonists. A consequence of activation of these receptors is an influx of calcium which is accumulated in the

mitochondria, and which then enhances free radical generation (Dykens, 1994).

MPTP is a neurotoxin which produces clinical, biochemical and neuropathologic changes in both human and non-human primates analogous to those which occur in Parkinson's disease (Bloem *et al.*, 1990). Its neurotoxic effects also appear to involve energy depletion and free radical generation. MPTP is converted to its metabolite 1-methyl-4-phenylpyridinium (MPP⁺) by monoamine oxidase B. MPP⁺ is selectively accumulated by the high affinity dopamine transporter and taken up into mitochondria of dopaminergic neurons, where it disrupts oxidative phosphorylation by inhibiting complex I of the mitochondrial electron transport chain (Tipton and Singer, 1993). This leads to impairment of ATP production, elevated intracellular calcium, and free radical generation (Sriram *et al.*, 1997; Hasegawa *et al.*, 1990).

If free radicals are involved in both malonate and MPTP neurotoxicity then free radical scavengers should exert neuroprotective effects. We previously showed that the free radical spin trap *N*-tert-butyl- α -(2-sulphophenyl)-nitron (S-PBN) dose-dependently protected against malonate lesions, and protected against a mild dosing regimen of MPTP that produced a 30% depletion of dopamine (Schulz *et al.*, 1995a, 1995b). A series of nitrones which are cyclic variants of α -phenyl-tert-butyl nitron (PBN) was recently described (Thomas *et al.*, 1994, 1996). The unsubstituted cyclic variant MDL 101,002 was approximately eightfold more potent than PBN in inhibiting lipid peroxidation, and 20–25 times more potent than PBN in trapping hydroxyl radicals (Fig. 1) (Thomas *et al.*, 1996). Tempol (4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl) is another promising free radical spin trap which may be particularly effective in scavenging peroxynitrite (Dikalov *et al.*, 1997). In the present experiments we examined whether MDL 101,002 and tempol could exert neuroprotective effects against malonate and MPTP neurotoxicity.

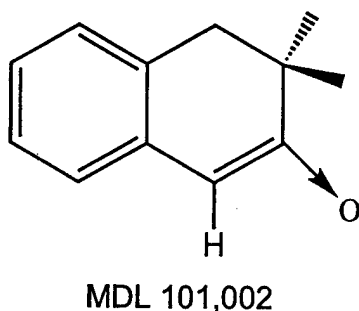


FIG. 1. Chemical structure of the cyclic α -phenyl-tert-butyl nitron derivative MDL 101,002.

MATERIALS AND METHODS

Malonate was obtained from Sigma (St. Louis, MO) and MPTP was obtained from Research Biochemicals (Wayland, MA). MDL 101,002 was generously supplied by Dr. Craig Thomas of Hoechst Marion Roussell. Male Sprague-Dawley rats (Charles River, Cambridge, MA) weighing 300–325 g were anesthetized with pentobarbital (50 mg/kg i.p.) and positioned in a David Kopf stereotaxic instrument with the incisor bar set at 3.3 mm below the interaural line. Intrastriatal injections of 3 μ mol of malonate in 1.5 μ l were made using a blunt-tipped 30-gauge Hamilton syringe as previously described (Beal, 1993). Ten animals in each group were treated with saline, 100 mg/kg of MDL 101,002 or 300 mg/kg of MDL 101,002 i.p. 30 min prior to malonate injections. Animals were sacrificed at 1 week and the brains were rapidly removed, placed in cold saline, and then sectioned coronally at 2-mm intervals. Slices were stained in 2% 2,3,5-triphenyltetrazolium chloride monohydrate (TTC) solution at room temperature for 30 min followed by fixation in phosphate-buffered 4% paraformaldehyde. The lesioned area was measured on the posterior surface of each section with a Bioquant IV image analysis system, and the total lesion volume was determined.

MPTP studies were carried out in male Swiss-Webster mice weighing 30–35 g (Taconic Farms, Germantown, NY). MPTP was administered in 0.1 ml water, pH adjusted to 7.4, at a dose of 15 mg/kg i.p. for five doses at 2-h intervals. Twelve animals in each group received normal saline, MPTP, or MPTP with 40 mg/kg of MDL 101,002 given with each dose of MPTP (200 mg/kg total). We also examined coadministration of the free radical spin trap tempol with MPTP at doses of 5, 10, or 20 mg/kg i.p. with each dose of MPTP. In this experiment MPTP was administered at 20 mg/kg i.p. for 4 doses at 2-h intervals. Animals were sacrificed at 1 week and the striata were rapidly dissected and placed in chilled 0.1 M perchloric acid. Tissue was subsequently sonicated and dopamine and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homova-

nillic acid (HVA) were measured by high performance liquid chromatography with 16-electrode electrochemical detection (Beal *et al.*, 1990).

To examine the effects of MDL 101,002 on 3-nitrotyrosine levels mice were treated with MPTP with or without MDL 101,002 as described above. Controls received normal saline. Mice were sacrificed 3 h after the last MPTP dose and the striata were dissected and placed in chilled 0.1 perchloric acid. Eight mice were examined in each group. The tissue was subsequently sonicated, centrifuged, and the supernatants were measured for 3-nitrotyrosine using high performance liquid chromatography with 16-electrode electrochemical detection as previously described (Schulz *et al.*, 1995d).

In a subsequent experiment, eight animals per group received either saline or 20 mg/kg of MDL 101,002 30 min prior to 30 mg/kg of MPTP. They were sacrificed 90 min after MPTP and the striata were dissected for measurements of MPP⁺ as previously described using HPLC with UV detection (Przedborski *et al.*, 1996). Similarly mice were treated with 30 mg/kg of MPTP and 10 mg of tempol and were sacrificed at 90 min for MPP⁺ levels.

Lesion volumes and neurochemical measurements are expressed as the mean \pm standard error of the mean. Statistical comparisons were made by one-way analysis of variance (ANOVA) followed by Fisher protected least significant difference (PLSD) post-hoc test to compare group means.

RESULTS

As shown in Fig. 2, pretreatment with MDL 101,002 produced neuroprotection against malonate lesions. The maximum neuroprotection was approximately 50%. Protection was also seen at a dose of 300 mg/kg, but this

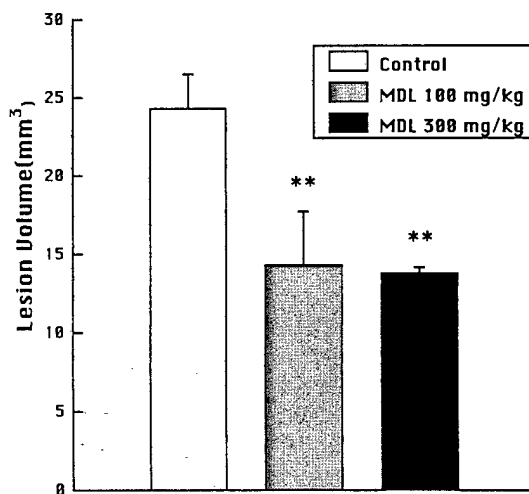


FIG. 2. Effects of pretreatment with MDL 101,002 on malonate-induced striatal lesions. ** $P < 0.01$ as compared with controls.

dose was toxic and only 5 of 10 treated animals survived.

The results of coinjection of MDL 101,002 on MPTP-induced dopamine depletion are shown in Fig. 3. MPTP produced approximately a 40% dopamine depletion as compared with controls. Coadministration of MDL 101,002 provided significant protection with dopamine depletion only 14%. MPTP also produced significant depletion of both DOPAC and HVA. With MDL 101,002, protection against the DOPAC depletion was not quite significant, but there was significant protection against the HVA depletion.

As shown in Fig. 4, MPTP administration resulted in a significant increase in 3-nitrotyrosine levels at 3 h after administration. Pretreatment with MDL 101,002 significantly attenuated the increases in 3-nitrotyrosine concentrations. MPP⁺ levels at 90 min after MPTP administration showed no significant difference between animals administered saline or MDL 101,002 (36.0 ± 4.5 vs 39.8 ± 9.7 ng/mg protein).

We examined the effects of tempol on a more severe dosing regimen of MPTP, which resulted in a 75% depletion of dopamine. The administration of tempol at doses of 5, 10, or 20 mg/kg i.p. with MPTP produced modest significant protection against MPTP induced dopamine depletion (Fig. 5). The protection with 5 mg/kg was slightly better than that seen with 10 or 20 mg/kg, and this dose also protected against DOPAC and HVA depletions. Administration of tempol at a dose of 20 mg/kg i.p. to normal mice exerted no toxic effects. MPP⁺ levels at 90 min after MPTP administration showed no significant difference between animals ad-

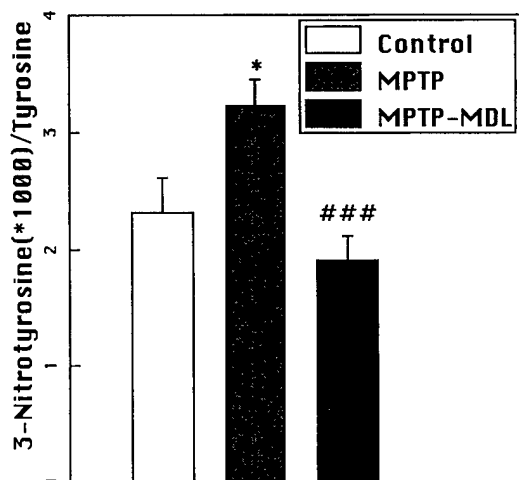


FIG. 4. Effects of treatment with MDL 101,002 on MPTP-induced increases in 3-nitrotyrosine concentrations at 3 h. * $P < 0.05$ compared with controls, ### $P < 0.001$ compared with MPTP.

ministered saline or 10 mg/kg of tempol (32.8 ± 2.5 vs 36.5 ± 4.3 ng/mg protein).

DISCUSSION

The pathogenesis of cell death in neurodegenerative diseases may involve a complex interaction between energy deficits, excitotoxicity, and free radical generation (Beal, 1997). Free radical spin traps such as PBN have been utilized to trap short-lived reactive radicals like $\cdot\text{OH}$ as the resultant nitroxide is a more stable

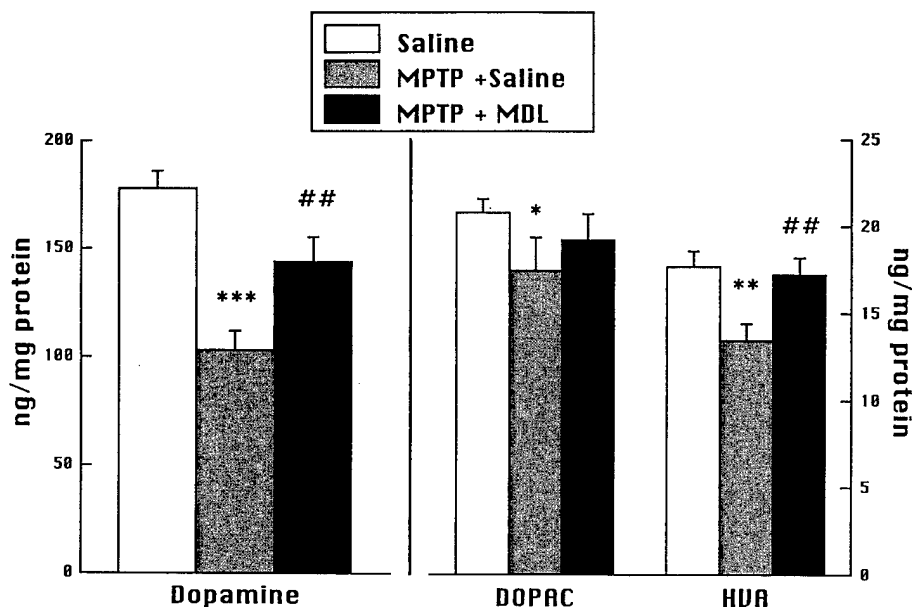


FIG. 3. Effects of treatment with MDL 101,002 on dopamine depletion produced by 5×15 mg/kg MPTP at 7 days. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with controls, ## $P < 0.01$ as compared with MPTP-treated mice.

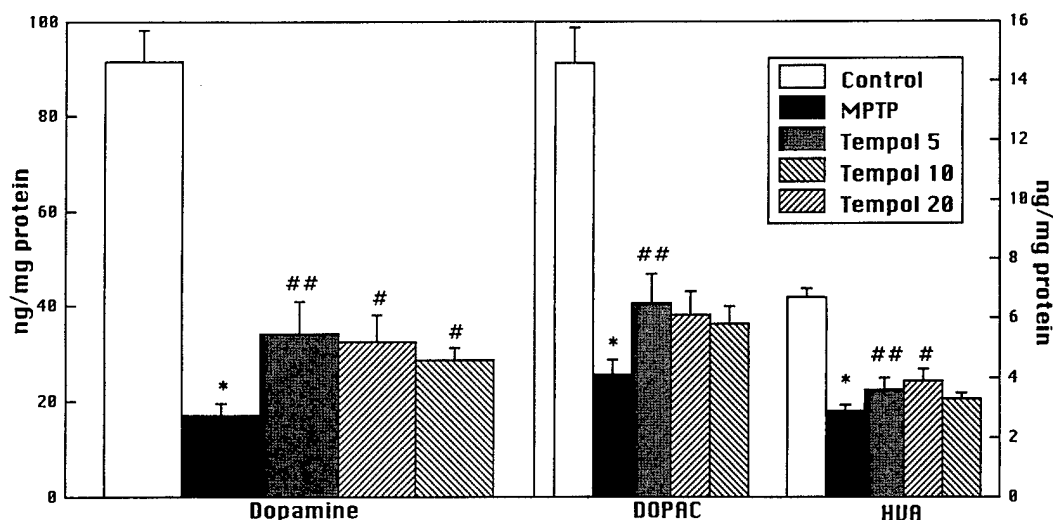


FIG. 5. Effects of treatment with tempol at 5, 10, or 20 mg/kg on dopamine depletion produced by 4×20 mg/kg MPTP at 7 days. * $P < 0.001$ as compared with controls, # $P < 0.05$, ## $P < 0.01$ as compared with MPTP-treated mice.

radical and can be detected using electron spin resonance spectroscopy (Knecht and Mason, 1993). Free radical spin traps exert neuroprotective effects against glutamate and NMDA toxicity *in vitro* (Yue *et al.*, 1992; Lafon-Cazal *et al.*, 1993). They are widely distributed in rats and penetrate the brain readily (Cheng *et al.*, 1993; Chen *et al.*, 1990). Of particular interest is their ability to concentrate in mitochondria, which may contribute to their therapeutic efficacy, since mitochondria are a major source of free radicals (Cova *et al.*, 1992). Prior work showed that PBN protects against age-associated accumulation of oxidative damage to proteins and cognitive deficits in gerbils (Carney and Floyd, 1991). Nitron spin trap treatment also extended survival and reduced oxidative damage to proteins in senescence accelerated mice (Edamatsu *et al.*, 1995; Butterfield *et al.*, 1997) and improved cognitive performance and survival of aging rats (Sack *et al.*, 1996).

Free radical spin traps show neuroprotective effects with various experimental paradigms. Several studies showed protection against ischemia/reperfusion-induced cerebral injury *in vivo* (Oliver *et al.*, 1990; Phillis and Clough-Helfman, 1990; Yue *et al.*, 1992). PBN showed protection when administered up to 12 h after permanent middle cerebral artery occlusion or up to 3 h after initiation of recirculation after transient middle cerebral artery occlusion (Cao and Phillis, 1994; Zhao *et al.*, 1994). Neuroprotective activity may be attributed to prevention of secondary deterioration of cellular bioenergetics attributed to mitochondrial dysfunction (Folbergrova *et al.*, 1995; Kuroda *et al.*, 1996) or attenuation of hydroxyl radical production associated with ischemia-reperfusion *in vivo* (Sen and Phillis, 1993). PBN also protects against methamphetamine-induced dopamine depletion, ecstasy-induced depletion of sero-

tonin, and seizure-induced loss of substantia nigra pars reticulata neurons (He *et al.*, 1997; Colado and Green, 1995; Cappon *et al.*, 1996). We previously showed that S-PBN can significantly attenuate excitotoxicity produced by *N*-methyl-D-aspartate, kainic acid, and AMPA in rat striatum (Schulz *et al.*, 1995c). Others showed that PBN protects against quinolinic acid neurotoxicity *in vivo* (Nakao *et al.*, 1996).

Earlier work demonstrated that malonate neurotoxicity was dose-dependently attenuated by S-PBN and that the protection was associated with reduced free radical generation as assessed by the conversion of salicylate to 2,3-dihydroxybenzoic acid (Schulz *et al.*, 1995b). In the present study we examined the effects of the cyclic analogue of PBN MDL 101,002. This compound shows an eightfold increase in potency against lipid peroxidation as compared with S-PBN, and a 20- to 25-fold increase in potency in trapping oxygen centered radicals (Thomas *et al.*, 1996, 1997). It is eightfold more potent than PBN in preventing oxidative injury to cerebellar granule cells *in vitro* (Thomas *et al.*, 1997). *In vivo* studies showed protection against ischemia/reperfusion in gerbils and against Fe^{2+} induced cortical injury (Thomas *et al.*, 1994, 1996, 1997).

In the present study, we found that MDL 101,002 produced significant protection against malonate induced striatal lesions. At a dose of 100 mg/kg protection was nearly maximal with approximately a 50% protection. This protection is greater than the 25% protection we achieved previously with 100 mg/kg of S-PBN, consistent with the greater *in vitro* potency of MDL 101,002 (Schulz *et al.*, 1995b). The brain concentration of MDL 101,002 following i.p. injection is similar to that reported for PBN (C. Thomas, unpublished data). A dose of 300 mg/kg of MDL 101,002 was associated with significant toxicity, and half the animals died.

We previously found that S-PBN exerts significant protection against a mild dosing regimen of MPTP producing a 30% depletion of dopamine (Schulz *et al.*, 1995a). Treatment with S-PBN however exerted no significant protection against MPTP regimens which resulted in 44 or 80% depletions of dopamine. In the present study MDL 101,002 produced significant protection against an MPTP dosing regimen which produced a 40% depletion of dopamine. This result therefore provides further evidence for improved *in vivo* efficacy of MDL 101,002 over that achieved with S-PBN.

We also examined the effects of tempol in an MPTP dosing regimen which produced a 75% dopamine depletion. Tempol reportedly can function as a metal-independent, membrane-permeable superoxide dismutase mimic (Mitchell *et al.*, 1990). Tempol inhibits radiation induced damage *in vitro* (Mitchell *et al.*, 1991) and it protects against radiation induced lethality in mice (Hahn *et al.*, 1992). It protects against hepatocyte injury resulting from the inhibition of mitochondrial respiration at low oxygen concentrations (Niknahad *et al.*, 1995) and against neutrophil and H₂O₂ mediated DNA damage (Hahn *et al.*, 1997). A recent study showed that it blocks hyperoxia-induced alterations in brain synaptosomal membranes showing that tempol crosses the blood-brain barrier (Howard *et al.*, 1996). Tempol is particularly interesting since it is chemically related to 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine (tempone-h), which is particularly reactive with peroxynitrite, showing a 10-fold higher sensitivity than two other spin traps (Dikalov *et al.*, 1997). Tempol produced significant protection at all three doses administered; however, maximal protection was observed with a dose of 5 mg/kg. We found no effect of tempol on MPP⁺ levels at 90 min; however, it remains possible that tempol and MDL 101,002 could attenuate MPTP-induced toxicity by limiting the bioavailability of the toxin at other time points.

The present results provide the first evidence that administration of free radical spin traps can attenuate increases in 3-nitrotyrosine induced by MPTP *in vivo*. We previously demonstrated that MPTP administration results in increases in 3-nitrotyrosine concentrations (Schulz *et al.*, 1995d) and that increases are blocked by the neuronal nitric oxide synthase inhibitor 7-nitroindazole. An involvement of nitric oxide and peroxynitrite in MPTP neurotoxicity is also strongly implicated by studies in neuronal nitric oxide synthase knockout mice and studies of nNOS inhibitors in baboons (Przedborski *et al.*, 1996; Hantraye *et al.*, 1996). It was recently shown that tyrosine hydroxylase is nitrated in the mouse striatum after MPTP administration and that this leads to a loss of enzyme activity (Ara *et al.*, 1998). In the present studies MDL 101,002 attenuated MPTP induced depletions of dopamine and increases in 3-nitrotyrosine, further implicating peroxy-

nitrite in the pathogenesis of MPTP neurotoxicity. MDL 101,002 could therefore be a direct scavenger of peroxynitrite or it could scavenge superoxide or nitric oxide preventing the formation of peroxynitrite.

Although free radical spin traps may directly scavenge intracellular free radicals several other potential mechanisms have been suggested. These include a metal-independent superoxide dismutase-like activity, stable oxidation of transition metal ions to reduce their ability to participate in Fenton chemistry, and inhibition of nitric oxide synthase induction (Krishna *et al.*, 1996; Miyajima and Kotake, 1997; Monti *et al.*, 1996). Regardless of mechanism the present studies provide further evidence for the involvement of free radicals in both malonate and MPTP neurotoxicity. Other work showed that malonate toxicity is attenuated by the free radical scavenger α -lipoic acid (Greenamyre *et al.*, 1994), and that MPTP neurotoxicity is attenuated in mice over-expressing superoxide dismutase (Przedborski *et al.*, 1992). By implication the present results provide further evidence that free radical scavengers may be effective in the treatment of neurodegenerative diseases such as Huntington's disease and Parkinson's disease.

ACKNOWLEDGMENTS

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1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine Neurotoxicity Is Attenuated in Mice Overexpressing Bcl-2

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The proto-oncogene Bcl-2 rescues cells from a wide variety of insults. Recent evidence suggests that Bcl-2 protects against free radicals and that it increases mitochondrial calcium-buffering capacity. The neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is thought to involve both mitochondrial dysfunction and free radical generation. We therefore investigated MPTP neurotoxicity in both Bcl-2 overexpressing mice and littermate controls. MPTP-induced depletion of dopamine and loss of [³H]mazindol binding were significantly attenuated in Bcl-2 overexpressing mice. Protection was more profound with an acute dosing regimen than with daily

MPTP administration over 5 d. 1-Methyl-4-phenylpyridinium (MPP⁺) levels after MPTP administration were similar in Bcl-2 overexpressing mice and littermates. Bcl-2 blocked MPP⁺-induced activation of caspases. MPTP-induced increases in free 3-nitrotyrosine levels were blocked in Bcl-2 overexpressing mice. These results indicate that Bcl-2 overexpression protects against MPTP neurotoxicity by mechanisms that may involve both antioxidant activity and inhibition of apoptotic pathways.

Key words: MPTP; Parkinson's disease; apoptosis; free radicals; 3-nitrotyrosine; caspases; Bcl-2

The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) results in a clinical syndrome closely resembling Parkinson's disease (PD) in both man and primates (Bloem et al., 1990). This meperidine analog is metabolized to 1-methyl-4-phenylpyridinium (MPP⁺) by the enzyme monoamine oxidase B. MPP⁺ is subsequently taken up selectively by dopaminergic terminals and concentrated in neuronal mitochondria in the substantia nigra pars compacta (SNpc). MPP⁺ binds to and inhibits complex I of the electron transport chain (Tipton and Singer, 1993). It may also cause irreversible inactivation of complex I by generating free radicals (Cleeter et al., 1992). MPP⁺ increases superoxide production in isolated bovine submitochondrial particles *in vitro* (Hasegawa et al., 1990) and *in vivo* (Sriram et al., 1997). MPTP-induced damage is attenuated in transgenic mice overexpressing superoxide dismutase, implicating free radical generation in its neurotoxicity (Przedborski et al., 1992).

The proto-oncogene Bcl-2 was initially characterized because of its ability to inhibit apoptosis. Bcl-2 is widely expressed in the nervous system and is localized to the outer mitochondrial membrane, endoplasmic reticulum, and nuclear membrane (Krajewski et al., 1993). Bcl-2 expression inhibits apoptosis in neural cells induced by a variety of stimuli (Bredesen, 1995). It also inhibits necrotic neural cell death in some paradigms, such as oxidative neural cell death induced by depletion of glutathione (Kane et al., 1995). Bcl-2 protects cells from the lethal effects of H₂O₂ or tertbutyl hydroperoxide in a dose-dependent manner (Hocken-

berry et al., 1993; Kane et al., 1993). It also protects neural cells from cyanide–aglycemia-induced lipid peroxidation, compromised mitochondrial respiration, and delayed cell death (Myers et al., 1995), as well as from AMPA toxicity in cortical cultures (White et al., 1997). It increases the capacity of neural cell mitochondria to accumulate calcium (Murphy et al., 1996). A critical role may be in regulation of membrane potential and volume homeostasis of mitochondria in response to apoptotic or necrotic stimuli (Vander Heiden et al., 1997). A recent study showed that Bcl-2 maintains the mitochondrial membrane potential, enhances H⁺ efflux after treatment with either Ca²⁺ or tertbutyl hydroperoxide, and prevents activation of the mitochondrial permeability transition (Shimizu et al., 1998).

Because mitochondrial dysfunction and oxidative injury play a role in the pathogenesis of MPTP neurotoxicity, we investigated whether MPTP neurotoxicity is attenuated in Bcl-2 overexpressing mice. The mice have a neuron-specific enolase (NSE) promoter fused to human Bcl-2 cDNA (Martinou et al., 1994). They overexpress Bcl-2 in multiple tissues, including the SNpc. Previous work showed that they are resistant to permanent ischemia induced by middle cerebral artery occlusion (Martinou et al., 1994) and that crossing them into a transgenic mouse model of amyotrophic lateral sclerosis extends survival (Kostic et al., 1997).

We examined the effects of both acute and chronic daily dosing regimen of MPTP in Bcl-2 overexpressing mice compared with littermate controls. Chronic (daily administration over 5 d) administration of MPTP induces apoptotic cell death in the SNpc of mice (Tatton and Kish, 1997), whereas no evidence of apoptosis was found with a more acute dosing regimen (Jackson-Lewis et al., 1995). We suspected that neuroprotection in Bcl-2 overexpressing mice would be more profound with a chronic dosing regimen. Surprisingly, there was almost complete protection

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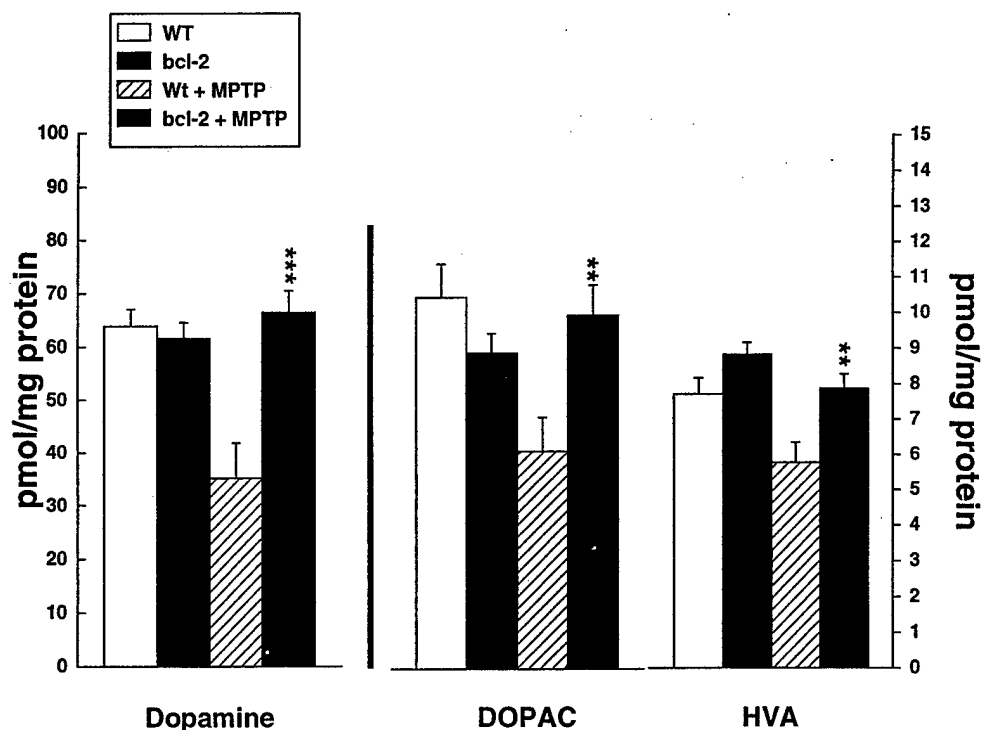


Figure 1. Effects of MPTP administered at 15 mg/kg intraperitoneally every 2 hr for 4 doses on dopamine, DOPAC, and HVA in wild-type and Bcl-2 overexpressing mice. ** $p < 0.01$; *** $p < 0.001$ compared with MPTP in controls.

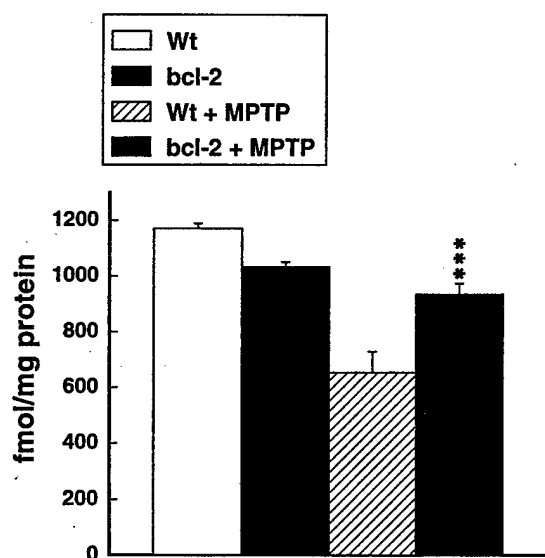


Figure 2. Effects of MPTP administered at 15 mg/kg intraperitoneally every 2 hr for 4 doses on [3H]mazindol binding in the striatum in wild-type and Bcl-2 overexpressing mice. *** $p < 0.001$ compared with MPTP in controls.

against MPTP neurotoxicity induced by an acute dosing regimen, whereas there was only partial protection with a chronic dosing regimen. We investigated the mechanism of neuroprotection in Bcl-2 overexpressing mice by showing that increases in 3-nitrotyrosine, a marker of oxidative damage, were attenuated and that caspase activation was inhibited.

MATERIALS AND METHODS

Human Bcl-2 overexpressing transgenic animals. Transgenic mice in which neurons overexpress the human Bcl-2 gene were generated using the NSE promoter by Martinou et al. (1994). We received male founders (strain NSE73A) and bred them with the same strain female mice to

obtain the hemizygous transgenic offsprings as assessed by PCR analysis of the DNA extracted from tissue taken from their tails. These mice are difficult to breed because females have an imperforate vagina and 50% of males are sterile. Wild-type littermates were used as controls.

MPTP in PBS was administered using either a chronic dosing regimen of 20 mg/kg intraperitoneally every 24 hr for five doses or an acute dosing regimen of 15 mg/kg intraperitoneally every 2 hr for four doses ($n = 10$ –12 mice in each group). Control animals in both paradigms were treated with a volume of PBS equal to the injection volume in the MPTP-treated animals. All animals in both paradigms were killed by decapitation 7 d after the last injection. For each mouse, one of the two striata was dissected, immediately frozen on dry ice, and stored at -80°C for measurement of dopamine and its metabolites. The other hemisphere was frozen in dry ice-cooled isopentane and sectioned on cryostat at $20\ \mu\text{m}$ for dopamine transporter ligand binding. The rest of the brain, including the mesencephalon, was placed into chilled 4% paraformaldehyde in PBS, fixed at 4°C for 24 hr, and then cryoprotected in 20% glycerol at 4°C .

Mice treated acutely with three doses of 15 mg/kg MPTP every 2 hr were killed at 3 and 6 hr after the last dose ($n = 6$ per group). The two striata were rapidly dissected and frozen at -80°C for MPP⁺ measurements. To evaluate the effects of MPTP on 3-nitrotyrosine levels, mice were injected with either saline or MPTP at 15 mg/kg intraperitoneally every 2 hr for four doses. Eight animals in each group were killed at 3 hr after the last dose (the time point at which we see a maximal increase in 3-nitrotyrosine levels after MPTP).

Stereological counts of tyrosine hydroxylase neurons. Eight Bcl-2 and seven littermate control mice were transcardially perfused with 4% buffered paraformaldehyde. Total neuron number in the SNpc was assessed using stereological principles. The nigra was serially sectioned, and every sixth section was immunostained with anti-tyrosine hydroxylase (TH). The number of TH-positive neurons was assessed using the optical disector technique and a systematic random sampling scheme using the stereology subroutines of Bioquant (Nashville, TN) Image Analysis Software. The volume of the SNpc was calculated by measuring the area on each section and using the Cavalieri principle.

Neurochemical analysis. Dissected striatal tissues were sonicated and centrifuged in 0.1 M perchloric acid (PCA) ($30\ \mu\text{L}/\text{mg}$ tissue). The supernatants were evaluated for levels of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), *p*-tyrosine, and 3-nitrotyrosine by HPLC with 16-electrode electrochemical detection as described previously (Beal et al., 1990). Concentrations of dopamine and metabolites are expressed as picomoles per milligram of

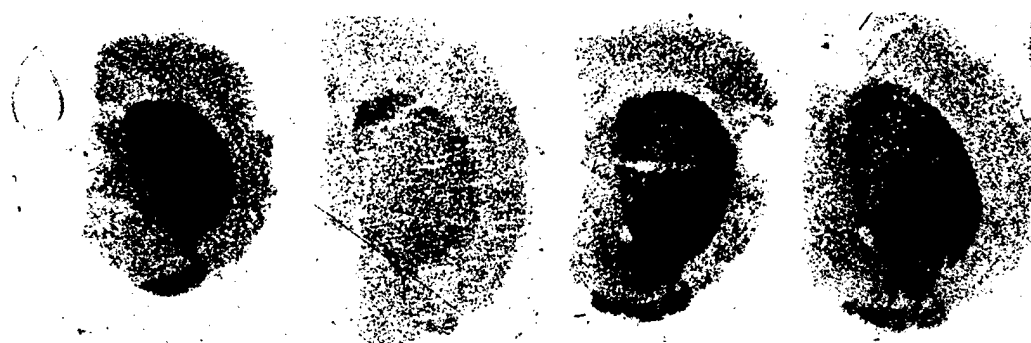


Figure 3. Representative autoradiographs of total [^3H]mazindol binding in the striatum in wild-type and Bcl-2 overexpressing mice after acute administration of MPTP. *Left*, Untreated wild-type mouse. *Middle-left*, MPTP-treated wild-type mouse. *Middle-right*, Untreated Bcl-2 transgenic mouse. *Right*, MPTP-treated Bcl-2 transgenic mouse.

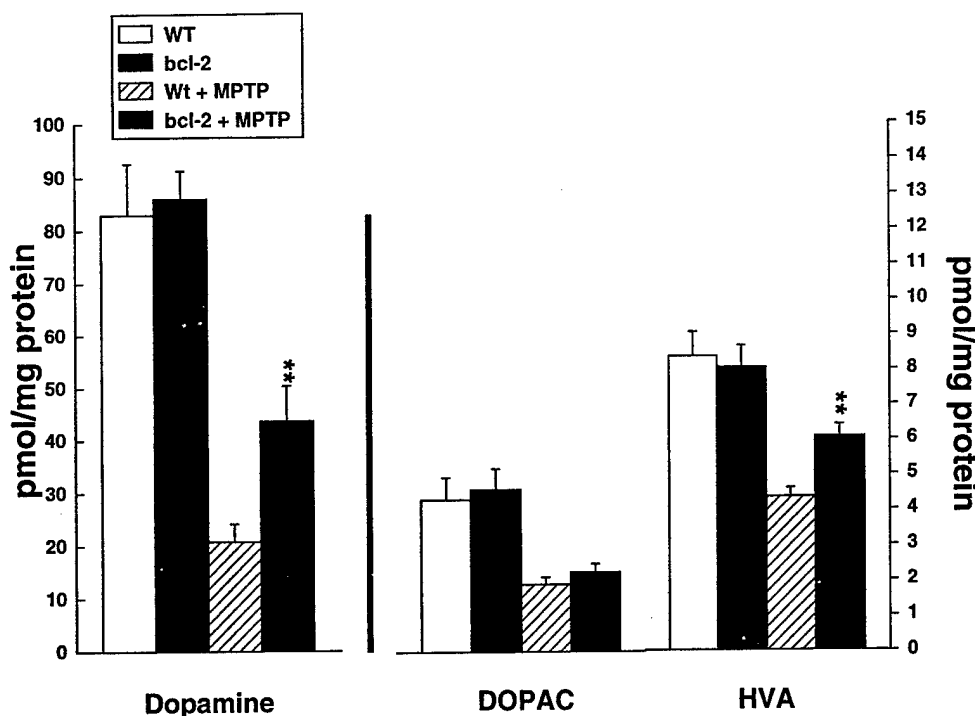


Figure 4. Effects of MPTP administered at 20 mg/kg intraperitoneally daily for 5 days on dopamine, DOPAC, and HVA in wild-type and Bcl-2 overexpressing mice. ** $p < 0.01$ compared with MPTP in controls.

protein. Free 3-nitrotyrosine data are expressed as ratios of 3-nitrotyrosine per *p*-tyrosine to normalize for differing brain concentrations of tyrosine. MPP⁺ levels (nanograms per milligram tissue wet weight) were quantified by HPLC with UV detection at 295 nm. Samples were sonicated in 0.1 M PCA, and an aliquot of supernatant was injected onto a Brownlee aquapore X03-224 cation exchange column (Rainin, Ridgefield, NJ). Samples were eluted isocratically with 90% 0.1 M acetic acid, 75 mM triethylamine-HCl, pH 2.35 adjusted with formic acid, and 10% acetonitrile. The flow rate was 1 ml/min.

Dopamine transporter binding autoradiography. Twenty micrometer striatal sections were prewashed 5 min in ice-cold buffer (50 mM Tris-HCl, 5 mM KCl, and 300 mM NaCl, pH 7.9) and then were incubated without drying in the ice-cold buffer containing 6 nM [^3H]mazindol and 300 nM desipramine for 60 min (nonspecific binding determined in the presence of 10 μM nomifensine) (Javitch et al., 1983). The slices were washed twice for 3 min in buffer chilled to 4°C and quickly dipped in cold distilled water. Then, they were hot-air dried and exposed to Hyperfilm- ^3H (Amersham, Arlington Heights, IL) at 4°C for 2 weeks. Films were developed with D19 (Eastman Kodak, Rochester, NY) developer. The films were analyzed with a video-based computerized image analysis system (MCID; Imaging Research, Inc., St. Catherine's, Ontario, Canada). The total striatal [^3H]mazindol binding (femtomoles per milligram of protein) was calculated using calibrated plastic ^{14}C standards (Penney et al., 1981; Pan et al., 1983).

Caspase activation. To examine for caspase activation, we injected MPP⁺, the active metabolite of MPTP, into the anterior striatum of wild-type and Bcl-2 overexpressing mice. MPP⁺ (Research Biochemicals, Wayland, MA) was dissolved in PBS at a concentration of 15 mM,

and 0.75 μl was injected. Four mice were killed at 12 and 24 hr after striatal MPP⁺ or saline, respectively. The striata were dissected from a 2-mm-thick slice and lysed on ice in 50 mM Tris-HCl, pH 8.0, containing 120 mM NaCl, 0.5% NP-40, 5 mM EDTA, 100 $\mu\text{g}/\text{ml}$ PMSF, 2 $\mu\text{g}/\text{ml}$ aprotinin, and 10 $\mu\text{g}/\text{ml}$ leupeptin, followed by centrifugation at 10,000 $\times g$ for 10 min. Samples were diluted to 1 $\mu\text{g}/\mu\text{l}$ protein and 20 $\mu\text{g}/\text{lane}$ subjected to SDS-PAGE on 12% polyacrylamide gels. After electrophoresis and electroblotting to nitrocellulose membranes, the blots were blocked in 250 mM Tris-HCl, pH 8.0, 120 mM NaCl, 10% nonfat dry milk, 5% BSA, 1% normal goat serum, 0.5% Tween 20, and 0.1% azide for 30 min. Next, the blots were incubated in the first antibody (anti-ICH-1_L; 1:1000; Transduction Laboratories, Lexington, KY) at 4°C overnight. After three washes in PBS (containing 0.05% Tween 20), the membranes were incubated with secondary alkaline phosphatase-conjugated antibody for 1 hr, washed three times in PBS, and stained with 0.2 mg/ml nitroblot tetrazolium chloride and 0.3 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate in 0.1 M Tris-HCl, pH 9.5, containing 50 mM MgCl_2 and 100 mM NaCl.

Statistical analysis. Statistical significance of differences between groups was determined via one-way ANOVA, followed by Fisher PLSD *post hoc* test to compare group means. The correlation of striatal [^3H]mazindol binding and dopamine levels was analyzed by Fisher's R to Z test.

RESULTS

Because NSE73A Bcl-2 mice have hypertrophic brains with increased numbers of neurons in some cell groups, we performed

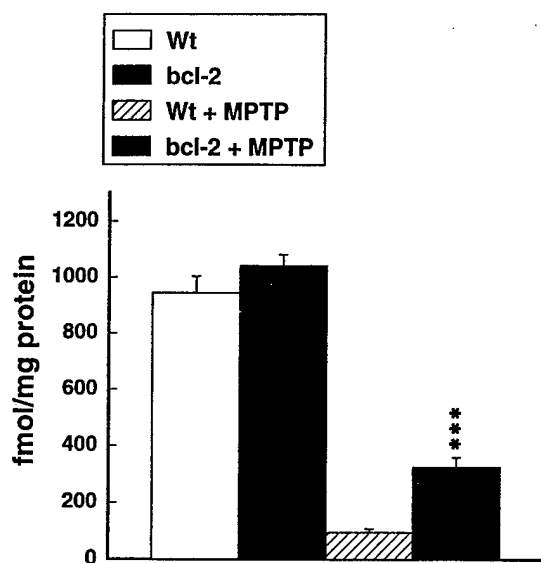


Figure 5. Effects of MPTP administered at 20 mg/kg intraperitoneally daily for 5 days on [3 H]mazindol binding in the striatum in wild-type and Bcl-2 overexpressing mice. *** $p < 0.001$ compared with MPTP in controls.

stereological cell counts of TH-immunopositive neurons in the SNpc of Bcl-2 overexpressing mice and littermate controls. Although there was a small increase in TH-immunopositive neurons in Bcl-2 mice compared with normal controls, the result was not significant. The number of TH-immunopositive neurons in the SNpc on one side in the controls was 5347 ± 433 , and the number was 5125 ± 265 ($p = 0.68$) in Bcl-2 overexpressing mice. Similarly, there were no significant differences in striatal dopamine levels of [3 H]mazindol binding at baseline (Figs. 1, 2).

The effects of MPTP administered acutely at a dose of 15 mg/kg intraperitoneally every 2 hr for 4 doses on dopamine, DOPAC, and HVA in wild-type (littermate) and Bcl-2 overexpressing mice are shown in Figure 1. There was significant, almost complete protection against depletions of dopamine, DOPAC, and HVA in the Bcl-2 overexpressing mice. Similarly, [3 H]mazindol binding in the striatum also showed almost complete protection (Fig. 2). Representative autoradiograms are shown in Figure 3.

The effects of administration of MPTP at a dose of 20 mg/kg daily for 5 consecutive days are shown in Figure 4. MPTP produced significant depletion of dopamine, DOPAC, and HVA. The depletions were significantly attenuated in the Bcl-2 overexpressing mice, but protection was not as complete as that seen with the acute dosing regimen. Similarly, [3 H]mazindol binding in the striatum showed partial significant protection that was not as profound as that seen with the acute dosing regimen (Fig. 5). Representative autoradiograms are shown in Figure 6.

MPP $^+$ levels were 3.74 ± 0.63 versus 4.56 ± 1.24 ng/mg wet weight at 3 hr in control and Bcl-2 mice, respectively ($p = 0.55$). At 6 hr, MPP $^+$ levels were 0.97 ± 0.57 and 0.53 ± 0.14 ng/mg wet weight in control and Bcl-2 overexpressing mice, respectively ($p = 0.48$). As shown in Figure 7, there was no difference in free 3-nitrotyrosine levels in Bcl-2 overexpressing mice compared with wild-type mice receiving saline. After administration of MPTP, there was a significant increase in free 3-nitrotyrosine levels in wild-type mice, which was significantly attenuated in Bcl-2 overexpressing mice.

By Western blot analysis, anti-ICH-1 $_L$ (Nedd2/caspase-2) rec-

ognized a major band at ~51 kDa in striatal lysates and, infrequently, a minor band at 45 kDa (Fig. 8). This apparent molecular weight of 51 kDa is in agreement with that calculated from the predicted sequence of the Nedd2 protein (Kumar et al., 1994; Harvey et al., 1997). MPP $^+$ induced an upregulation of this protein at 12 and 24 hr in wild-type animals and to a lesser extent in Bcl-2 overexpressing mice. Further, a cleavage product of ~24 kDa was detectable by Western blotting in wild-type animals but not in Bcl-2 mice.

DISCUSSION

Bcl-2 is a protein that inhibits both apoptotic and necrotic cell death. Although the specific mechanism of action of Bcl-2 is unknown, it can either detoxify or decrease the production of reactive oxygen species (Kane et al., 1993; Hockenberry et al., 1993; Lawrence et al., 1996). There is a direct antioxidant effect of Bcl-2 in PC12 rat pheochromocytoma cells (Tyurina et al., 1997). Neural cells expressing Bcl-2 have elevated levels of reduced glutathione/oxidized glutathione and NADH/NAD $^+$, indicating a shift in cellular redox potential to a more reduced state (Ellerby et al., 1996). Bcl-2 causes a redistribution of glutathione to the nucleus (Voehringer et al., 1998). Bcl-2 also has beneficial effects on mitochondrial function. It enhances the mitochondrial membrane potential and improves ATP/ADP ratios (Hennet et al., 1993; Smets et al., 1994) and delays ATP depletion induced by growth factor withdrawal (Garland and Halestrap, 1997). Overexpression of Bcl-2 enhances the mitochondrial calcium uptake potential of neural cells (Murphy et al., 1996), and it inhibits mitochondrial release of calcium (Baffy et al., 1993). Bcl-2 expression inhibits the mitochondrial transition pore and release of an apoptogenic protease (Susin et al., 1996; Zamzami et al., 1996; Shimizu et al., 1998). Bcl-2 expression also blocks the release of cytochrome c from mitochondria (Kluck et al., 1997; Yang et al., 1997), which is linked to apoptosis (Liu et al., 1996). The related protein Bcl-x $_L$ also blocks cytochrome c release by directly binding to cytochrome c (Kharbanda et al., 1997) and by blocking rupture of the outer mitochondrial membrane (Vander Heiden et al., 1997). Bcl-2 targets the protein kinase raf-1 to mitochondria where it helps to block apoptosis (Wang et al., 1996). In other studies, it blocks the activation of caspases, indicating that it acts upstream of caspases in the cell death pathway (Chinnaiyan et al., 1996; Srinivasan et al., 1996). Bcl-2, as well as the Bcl-2 analog Bcl-x $_L$, forms ion channels in synthetic lipid membranes (Minn et al., 1997; Schendel et al., 1997), and it inhibits Bax channel-forming activity (Antonsson et al., 1997). Bcl-x $_L$ inhibits mitochondrial swelling, regulates membrane potential in response to both necrotic and apoptotic stimuli (Vander Heiden et al., 1997), and inhibits a loss of mitochondrial membrane potential by regulating proton flux (Shimizu et al., 1998).

Substantial evidence implicates mitochondrial dysfunction and free radical generation in MPTP neurotoxicity. Because Bcl-2 expression can modify both of these processes, we examined whether MPTP neurotoxicity is reduced in mice overexpressing Bcl-2. Both necrotic and apoptotic cell death mechanisms may play a role in MPTP neurotoxicity, depending on the severity of the insult. In PC12 cells, a high dose of MPP $^+$ induces rapid necrotic cell death, whereas lower doses produce delayed apoptotic cell death (Hartley et al., 1994). MPTP administered at a dose of 20 mg/kg for 5 d induced apoptotic cell death in the SNpc, as documented using both *in situ* end labeling with terminal deoxynucleotidyl transferase and staining for chromatin condensation with acridine orange (Tatton and Kish, 1997). In contrast, an

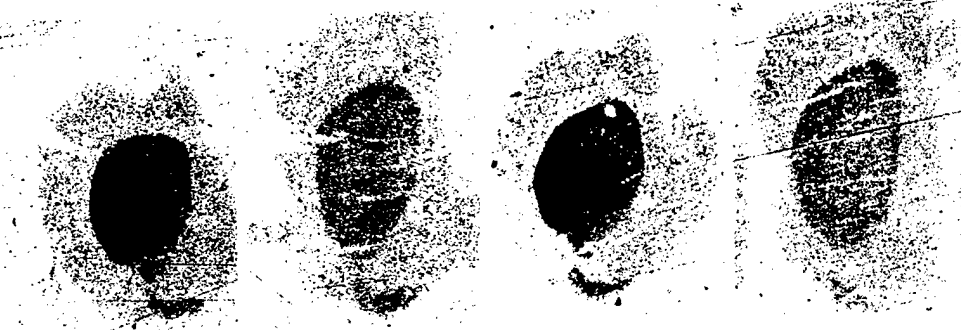


Figure 6. Representative autoradiographs of total [^3H]mazindol binding in the striatum in wild-type and Bcl-2 overexpressing mice after chronic administration of MPTP. Left, Untreated wild-type mouse. Middle, MPTP-treated wild-type mouse. Midright, Untreated Bcl-2 transgenic mouse. Right, MPTP-treated Bcl-2 transgenic mouse.

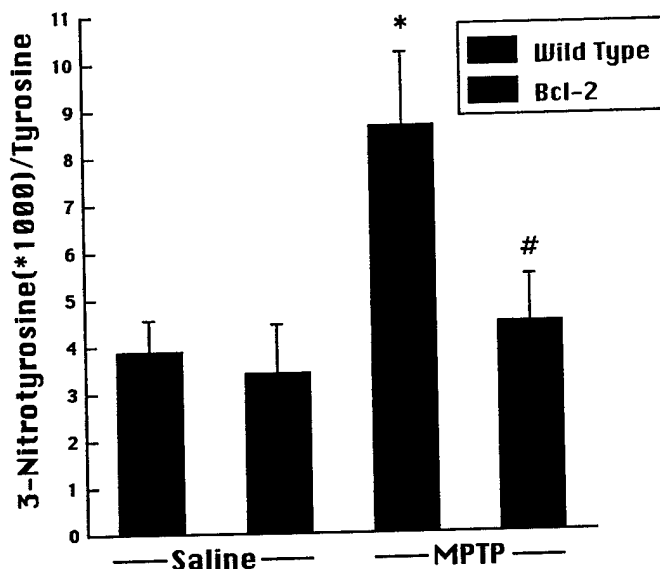


Figure 7. Effects of MPTP administration on 3-nitrotyrosine concentrations in wild-type and Bcl-2 overexpressing mice. MPTP induced a significant increase in 3-nitrotyrosine at 3 hr after the last dose compared with saline treated controls, which was significantly attenuated in Bcl-2 overexpressing mice. * $p < 0.05$ compared with saline; # $p < 0.05$ compared with MPTP-treated wild type.

acute dosing regimen of MPTP did not show evidence of apoptotic nuclei (Jackson-Lewis et al., 1995). We therefore hypothesized that an acute dosing regimen of MPTP would be more likely to induce necrotic cell death, and a more chronic dosing regimen administered daily would be more likely to induce apoptotic cell damage.

In the present experiments, to our surprise, there was almost complete protection from MPTP-induced decreases in dopamine levels and [^3H]mazindol binding induced by acute administration of MPTP in mice overexpressing Bcl-2. Although the fall in [^3H]mazindol binding seen after MPTP lesions could represent downregulation of dopamine transporter numbers or affinities in surviving dopamine terminals, the fact that MPTP is known to kill dopamine neurons makes it most likely that this result represents protection of dopamine neurons and their terminals by Bcl-2. There was also complete protection against MPTP-induced depletions of the dopamine metabolites DOPAC and HVA. The neuroprotective effects were not attributable to an alteration in numbers of substantia nigra neurons, as shown by stereological cell counts of TH neurons. After chronic daily administration of MPTP, there was significant partial protection against depletions of dopamine, HVA, and [^3H]mazindol binding; however, it was much less marked than the protection seen

with the acute dosing regimen. These results indicate that Bcl-2 protects against both acute and chronic dosing regimens of MPTP neurotoxicity, but it is much more effective against an acute dosing regimen in which necrotic cell death would be expected to predominate. This is of course unexpected, because Bcl-2 is well known to have anti-apoptotic properties. However, if a primary mechanism is to stabilize mitochondria and induce antioxidant effects, one might well expect protection against both necrotic and apoptotic cell damage.

The mechanism of neuroprotective effects of Bcl-2 overexpression was investigated. There were no effects on MPP $^+$ levels in the Bcl-2 expressing mice compared with littermate controls, indicating that the neuroprotective effects of Bcl-2 are not mediated by an alteration in MPTP uptake or metabolism to MPP $^+$. There also were no significant differences in [^3H]mazindol binding in Bcl-2 expressing mice compared with littermate controls at baseline, indicating no change in the dopamine transporter.

Recent evidence indicates that Bcl-2 acts upstream of caspase proteases in programmed cell death to inhibit their activation. Bcl-2 blocks release of cytochrome c and subsequent activation of caspases *in vitro* (Kluck et al., 1997; Yang et al., 1997). In the cytosol, cytochrome c binds to apoptotic protease activating factor-1, the mammalian homolog of CED-4, which may trigger the activation of caspase-3 (Zou et al., 1997). After MPP $^+$ injections, our extracts show a major band at 51 kDa, agreeing with the predicted molecular weight of Nedd2 (Kumar et al., 1994; Harvey et al., 1997). This probably reflects damage to both dopaminergic terminals and intrinsic striatal neurons, because MPP $^+$ injections result in striatal damage (Storey et al., 1994). Striatal MPP $^+$ injections lead to an upregulation of this protein, which was reduced in Bcl-2 overexpressing mice. Activation of Nedd2 requires the cleavage of the 51 kDa precursor molecule into subunits of 19 and 12 kDa (Harvey et al., 1997). Cleavage of Nedd2 has been reported in other neuronal cell death paradigms, namely in the trophic factor withdrawal-induced apoptosis of differentiated PC12 cells (Troy et al., 1997) and staurosporine-induced apoptosis of neuronal GT1-7 cells (Srinivasan et al., 1996). At 12 and 24 hr after injection of MPP $^+$ into the striatum, a cleavage product of ~24 kDa was detectable in wild-type, but not in Bcl-2 transgenic, animals. At present, we cannot explain the discrepancy between the predicted molecular weight of the cleavage products (12 and 19 kDa) and the observed 24 kDa band. Because this cleavage product only occurred after upregulation of Nedd2 protein and the cleavage was blocked by Bcl-2, we feel that it represents a cleaved subunit of Nedd2. Possibly, this cleavage product is an intermediate form of the 19 or 12 kDa subunit.

Although neuronal Bcl-2 overexpression did not block upregulation of Nedd2 protein, the cleavage of Nedd2 into active subunits was completely blocked, consistent with recent results

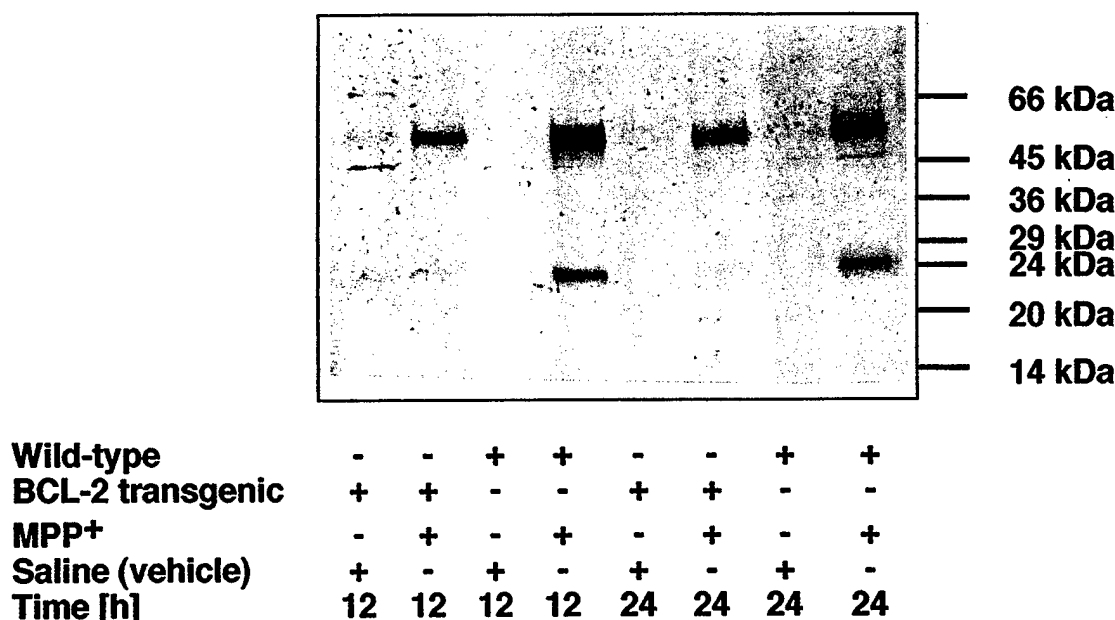


Figure 8. Striatal MPP⁺ injections induce Bcl-2-sensitive activation of caspase-2. MPP⁺ or vehicle (saline) were injected into the striatum of wild-type or Bcl-2 transgenic animals, and immunoblot analysis of striatal lysates was performed at 12 and 24 hr after injection. The 51 kDa band corresponds to caspase-2 (Nedd2/ICH-1_L), and the 24 kDa band represents a cleaved product of caspase-2.

(Srinivasan et al., 1996) showing that Bcl-2 blocks apoptosis by preventing processing of the proforms of caspases into the active forms. These findings therefore provide *in vivo* evidence linking MPP⁺ to caspase activation and showing that Bcl-2 acts upstream to prevent this activation.

We also examined whether overexpression of Bcl-2 could inhibit MPTP-induced oxidative damage *in vivo*. We showed previously that MPTP neurotoxicity is associated with increases in striatal concentrations of free 3-nitrotyrosine, a marker of oxidative damage mediated by peroxynitrite (Schulz et al., 1995). Furthermore, we and others found that neuronal nitric oxide synthase inhibitors, which block the generation of peroxynitrite, produce neuroprotection against MPTP neurotoxicity in both mice and primates (Schulz et al., 1995; Hantraye et al., 1996; Przedborski et al., 1996). In the present study, we found that MPTP-induced increases in free 3-nitrotyrosine were significantly attenuated in mice overexpressing Bcl-2. These data therefore provide *in vivo* evidence that one mechanism of the neuroprotective effects of Bcl-2 is by inhibiting oxidative damage.

Our results are consistent with recent studies that showed that expression of Bcl-2 can inhibit lipid peroxidation and cyanide-aglycemic-induced cell death *in vitro* (Myers et al., 1995). Overexpression of Bcl-2 with herpes simplex vectors enhances neuronal survival in cultured neurons exposed to glutamate and hypoglycemia and protects against focal ischemia in the striatum (Lawrence et al., 1996). Our results are also consistent with the finding that permanent focal ischemic lesions are attenuated in Bcl-2 overexpressing mice and that neurons that survive ischemic lesions *in vivo* show upregulation of Bcl-2 (Martinou et al., 1994; Chen et al., 1995). Overexpression of Bcl-2 also prolongs survival and attenuates motor neuron degeneration in a transgenic animal model of amyotrophic lateral sclerosis (Kostic et al., 1997).

The present results suggest that expression of Bcl-2 or administration of Bcl-2 mimics might be useful in the treatment of PD. Evidence implicating apoptosis in PD is controversial. Some studies found evidence for apoptosis based on morphological

criteria or *in situ* end labeling (Mochizuki et al., 1996; Anglade et al., 1997), whereas others did not (Kosel et al., 1997). An increase in Bcl-2 protein was found in caudate and putamen of PD patients (Mogi et al., 1996). Whether neuronal death in PD occurs by either apoptosis or necrosis, our present results suggest that Bcl-2 may exert neuroprotective effects. Furthermore, recent evidence indicates that it can promote regeneration of severed retinal axons *in vitro*, independent of its anti-apoptotic effects (Chen et al., 1997). This suggests that Bcl-2 might exert both neuroprotective effects, as well as restorative effects, in promoting regrowth of dopaminergic axons in PD.

Addendum

While this manuscript was in review, others have found that Bcl-2 overexpressing mice are protected against acute MPTP-induced dopamine depletion (Offen et al., 1998).

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